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sushovan.guha@uth.tmc.edu

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REVIEW

Emerging roles of non-coding RNAs in colorectal cancer oxaliplatin resistance and liquid biopsy potential

Zheng-Dong Luo, Yi-Feng Wang, Yu-Xiao Zhao, Long-Chen Yu, Tian Li, Ying-Jing Fan, Shun-Jie Zeng, Yan-Li Zhang, Yi Zhang, Xin Zhang

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Zheng-Dong Luo, Yi-Feng Wang, Yu-Xiao Zhao, Long-Chen Yu, Tian Li, Ying-Jing Fan, Shun-Jie Zeng, Yi Zhang, Xin Zhang, Department of Clinical Laboratory, Qilu Hospital of Shandong University, Shandong Engineering Research Center of Biomarker and Artificial Intelligence Application, Jinan 250012, Shandong Province, China

Yan-Li Zhang, Department of Clinical Laboratory, Shandong Provincial Third Hospital, Jinan 250012, Shandong Province, China

Corresponding author: Xin Zhang, MD, PhD, Additional Professor, Department of Clinical Laboratory, Qilu Hospital of Shandong University, Jinan 250012, Shandong Province, China. xinzhang@sdu.edu.cn

Abstract

Colorectal cancer (CRC) is one of the most common malignancies of the digestive tract, with the annual incidence and mortality increasing consistently. Oxaliplatin-based chemotherapy is a preferred therapeutic regimen for patients with advanced CRC. However, most patients will inevitably develop resistance to oxaliplatin. Many studies have reported that non-coding RNAs (ncRNAs), such as microRNAs, long non-coding RNAs, and circular RNAs, are extensively involved in cancer progression. Moreover, emerging evidence has revealed that ncRNAs mediate chemoresistance to oxaliplatin by transcriptional and post-transcriptional regulation, and by epigenetic modification. In this review, we summarize the mechanisms by which ncRNAs regulate the initiation and development of CRC chemoresistance to oxaliplatin. Furthermore, we investigate the clinical application of ncRNAs as promising biomarkers for liquid CRC biopsy. This review provides new insights into overcoming oxaliplatin resistance in CRC by targeting ncRNAs.

Key Words: Colorectal cancer; Non-coding RNAs; Oxaliplatin; Resistance; Liquid biopsy biomarkers

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Core Tip: Oxaliplatin has served as a first-line chemotherapy option for colorectal cancer (CRC). However, owing to congenital or acquired resistance, treatment failure is common in some patients with CRC. Abundant evidence has revealed that non-coding RNAs (ncRNAs) are extensively involved in cancer progression, including drug resistance. Specifically, ncRNAs mediate resistance to oxaliplatin by mediating drug carriers, tumor microenvironment, resistance-related signaling pathways, and patterns of cell death. Importantly, we investigated the potential and clinical application values of these ncRNAs as liquid biopsy markers for CRC.

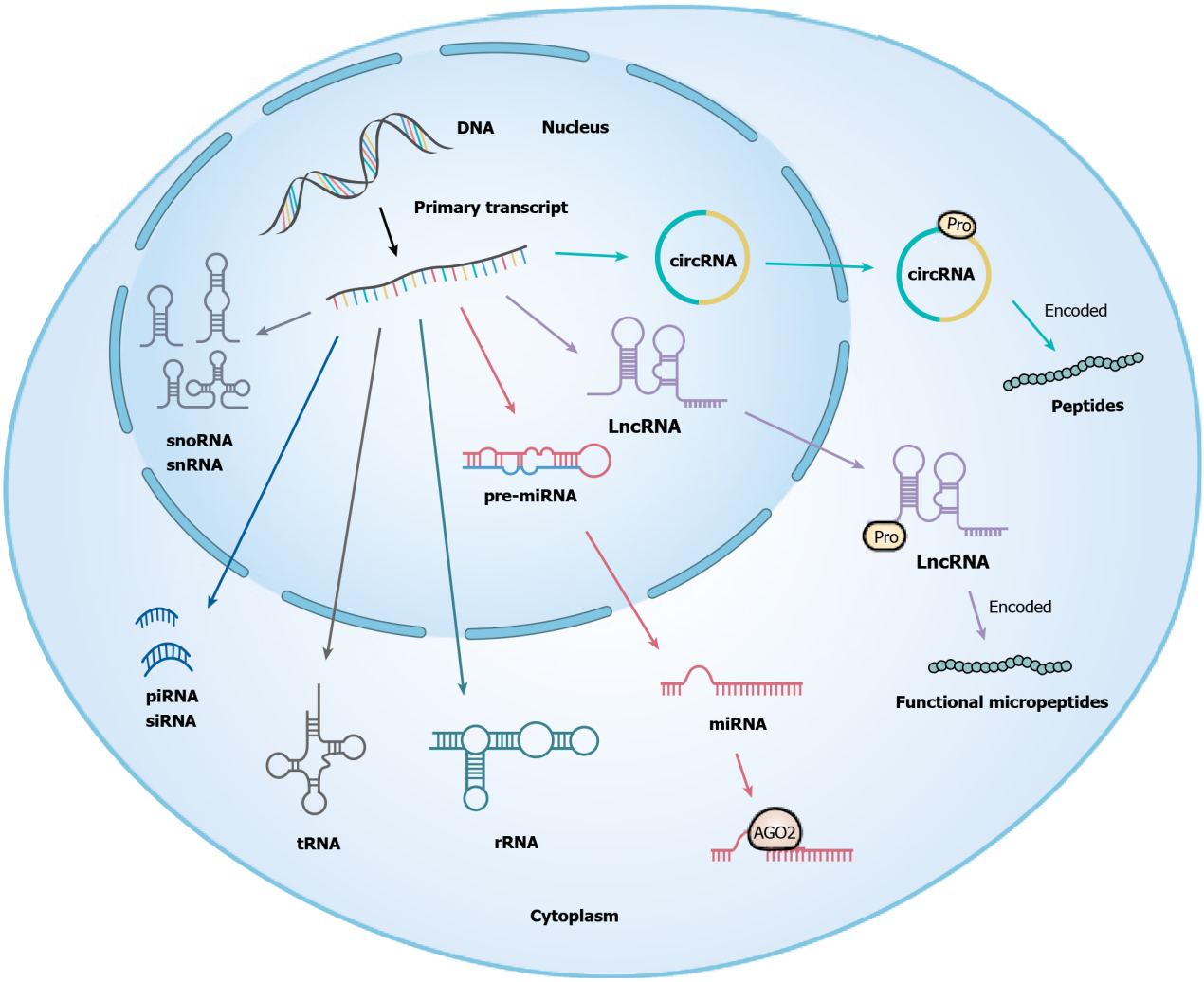
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INTRODUCTION

Colorectal cancer (CRC) is considered to be the leading cause of death associated with malignancy of the gastrointestinal tract, with approximately 1932 million new cases and 935000 deaths in 2020[1]. Currently, surgery is the preferred treatment option for early CRC patients; however, it has few clinical benefits for advanced patients due to high rates of postoperative metastasis and recurrence[2,3]. The annual survival rate for stage III CRC patients is about 30%-60%; this figure drops to just 10% for stage IV patients[4,5]. The combination of oxaliplatin, 5-fluorouracil, and leucovorin (FOLFOX) has significantly increased the overall survival of patients and now serves as a first-line standard regimen for metastatic CRC (mCRC)[6]. Oxaliplatin is a widely used third-generation platinum analog that functions by reacting with DNA to form hydration derivatives with intra- and inter-strand crosslinks eventually resulting in cell death[7]. However, oxaliplatin has not been satisfactory in improving the survival rate of some patients due to drug resistance[8]. Moreover, some toxic side effects persist after oxaliplatin treatment; these include peripheral neurotoxicity, which further hinders chemotherapy[9]. Fortunately, oxaliplatin-based chemotherapy retreatment strategies are being optimized to provide further personalized therapy for patients with mCRC[10]. Therefore, it is of great importance to understand the mechanism by which oxaliplatin resistance occurs, and to identify new biomarkers that can aid prediction of the outcomes for CRC patients treated with oxaliplatin-based therapy.

Although the processes of chemoresistance in CRC are intricate and inconclusive[11], ongoing research has revealed multiple oxaliplatin resistance mechanisms. Several studies have confirmed that chemoresistance is associated with the dysregulation of efflux proteins and drug-metabolizing enzymes, such as ABC transporters and GSTP1; these enzymes mediate drug uptake, transport, and toxicity[12,13]. Furthermore, accumulating evidence suggests that epithelial-mesenchymal transition (EMT)[14] and DNA damage repair[15] are driving factors that result in chemoresistance and tumor progression. Many studies have reported that various signaling pathways, such as the TGF-β/Smad[16], JNK/p38 MAPK[17], Wnt/β-catenin[18], and MEK/ERK/ELK1[19] pathways are closely associated with oxaliplatin resistance. As the main regulatory components of the tumor microenvironment (TME), cancer-associated fibroblasts (CAFs)[20] and tumor-associated macrophages[21] can modulate cancer chemotherapy resistance through complex mechanisms of crosstalk. Of note, emerging studies have confirmed that cell death mechanisms play a pivotal role in chemoresistance. It has been reported that apoptosis inhibition[22] and autophagy dysregulation[23] are underlying mechanisms leading to chemoresistance. Furthermore, numerous studies have identified that several additional death mechanisms can mediate CRC chemoresistance, including ferroptosis[24], pyroptosis[25], necroptosis[26], and others. However, the detailed molecular mechanisms by which these cell death modes lead to oxaliplatin resistance are unclear and require further investigation.

Non-coding RNAs (ncRNAs) refer to RNA molecules transcribed from genes that cannot encode proteins, accounting for more than 90% of human gene transcripts. ncRNAs can be divided into either small ncRNAs (< 200 nucleotides) or long non-coding RNAs (lncRNAs) (> 200 nucleotides) based on their length[27-29]. In the last decade, circular RNAs (circRNAs), a type of circular RNA generated in the splicing process of pre-mRNA, have emerged as a focus of research[30] (Figure 1). A growing number of studies have revealed that ncRNAs play critical roles in the occurrence and development of multiple human diseases *via* various mechanisms, such as epigenetics, transcription, and post-transcription[31,32]. Moreover, microRNA (miRNAs), lncRNAs, and circRNAs are three of the more widely studied types of ncRNAs and have been shown to mediate tumor progression by regulating tumor cell proliferation, aggression, metastasis, apoptosis, and drug resistance, amongst other pathways [33,34]. Drug resistance is one of the key factors in malignancy treatment failure, and whether this is mediated by ncRNAs has attracted increasing attention. In recent years, the rapid development of next-



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Figure 1 Classification of non-coding RNAs. Non-coding RNAs can be divided into either small ncRNAs (< 200 nucleotides) or long non-coding RNAs (> 200 nucleotides) based on their length. Among these, small ncRNAs consist of ribosomal RNAs, transfer RNAs, small nucleosome/spliced RNAs, microRNAs, small interfering RNAs, PiWI-Interacting RNAs, and small nucleolar RNAs. Furthermore, the circular RNAs generated in the splicing process of pre-mRNA are also considered to be special ncRNAs. ncRNAs: Non-coding RNAs; lncRNAs: Long non-coding RNAs; rRNAs: Ribosomal RNAs; tRNAs: Transfer RNAs; snRNAs: Small nucleosome/spliced RNAs; miRNAs; MicroRNAs; siRNAs: Small interfering RNAs; piRNAs: PiWI-Interacting RNAs; snoRNAs: Small nucleolar RNAs.

generation sequencing technologies has expanded the understanding of tumor pathogenesis and drug resistance mechanisms, and an increasing number of tumor chemoresistance-associated differential ncRNAs have been identified[35-37]; these developments have provided the expansion of open datasets of candidate ncRNAs for basic research and clinical application. Currently, the targeting of ncRNAs as biomarkers in the clinic is being actively facilitated[38]. As such, a systematic understanding of the underlying roles of ncRNAs in malignancies provides valuable insights into overcoming chemoresistance in CRC patients.

Although tissue biopsy is the gold standard for malignancy diagnosis, the availability of clinical samples is often limited, and subsequently, tumor heterogeneity may not be reflected in analysis and is therefore not suitable for longitudinal clinical monitoring[39]. As a noninvasive approach, liquid biopsy has been extensively applied for the real-time monitoring of variations in tumor dynamics in body fluids including blood, ascites, and others. With the development of novel molecular detection technologies, growing evidence suggests that circulating tumor cells, circulating tumor DNA, circulating free nucleic acids, tumor-educated platelet, exosomes, *etc*[40,41] have gradually become prominent liquid biopsy hallmarks. Owing to their stability and high abundance, circulating ncRNAs can provide substantial details regarding tumor biology and therapeutic efficacy, and have become candidate biomarkers for earlier diagnosis, therapeutic monitoring, and prognosis evaluation of malignancies[42,43].

In this study, we performed a systematic literature review to identify the latent mechanisms of ncRNAs (miRNAs, lncRNAs, and circRNAs) in CRC oxaliplatin resistance. In particular, exosomal ncRNAs, as one of the most promising biomarkers in liquid biopsies, are expected to improve diagnostic, therapeutic, and drug monitoring in CRC patients.

OVERVIEW OF THE REGULATORY ROLES OF NCRNAS IN CRC OXALIPLATIN RESISTANCE

Classification and biological functions of miRNAs

MiRNAs are a class of small ncRNAs of approximately 22 nucleotides in length; they are produced by two RNase III proteins, Drosha and Dicer[44]. In general, miRNAs bind to the 3' untranslated region (UTR) of their target mRNAs resulting in cleavage or translational repression in the cytosol[45]. Intriguingly, numerous recent studies have demonstrated that some miRNAs activate gene transcription unconventionally in the nucleus by targeting enhancers, such miRNAs are known as nuclear activating miRNAs[46]. Moreover, enhancers (including super-enhancers) have been revealed to synergistically promote NamiRNA biogenesis and activate the expression of proximal genes[47,48]. Furthermore, studies have reported that the dysregulation of miRNAs may play an important role in CRC oxaliplatin resistance, as illustrated in Table 1.

Oncogenic miRNAs mediate oxaliplatin resistance in CRC

MiR-135b-5p has been confirmed to be upregulated in the serum of CRC patients, mechanistically this has been shown to induce protective autophagy *via* the MUL1/ULK1 signaling pathway, a process that contributes to oxaliplatin resistance[49]. Another miRNA, miR-454-3p, is highly expressed in oxaliplatin-resistant CRC cells compared to oxaliplatin-sensitive cells and promoted oxaliplatin resistance by inhibiting PTEN expression and activation of the AKT pathway[50]. Similarly, targeting miR-19a with high expression in oxaliplatin-resistant CRC cell lines could promote oxaliplatin sensitization of resistant cells through activation of the PTEN/PI3K/AKT pathway[51]. Compounding these findings, it has been revealed that dichloroacetate, a pyruvate dehydrogenase kinase inhibitor, can enhance the chemosensitivity of oxaliplatin-resistant CRC cells through the miR-543/PTEN/Akt/mTOR pathway and the miR-107/CAB39/AMPK/mTOR pathway[52,53]. In addition, an unbiased microRNA array demonstrated that miR-503-5p was up-regulated in oxaliplatin-resistant CRC cells; overexpression of miR-503-5p conferred resistance to oxaliplatin-induced apoptosis by inhibiting PUMA expression[54]. Further studies have revealed that by targeting BNI1, up-regulation of miR-744 enhanced the resistance of T84 and HCT116 cells to oxaliplatin[55]. Moreover, the oncogenic miR-5000-3p was upregulated in CRC tissues and oxaliplatin-resistant CRC cells, this miRNA negatively regulated USP9 to facilitate CRC chemoresistance[56]. Additionally, natural killer cells contributed to enhancing the sensitivity of oxaliplatin resistant CRC cells through the microRNA-146b-5p/WBSCR22 axis[57]. Remarkably, miR-19b-3p has been documented to be the most significantly upregulated candidate miRNA in colon cancer tissues, its expression correlates with tumorous histologic grading, staging, and poor prognosis in patients. Importantly, miR-19b-3p facilitated proliferation, curbed apoptosis, and induced oxaliplatin resistance in CRC cells by targeting SMAD4[58].

Tumor-suppressive miRNAs mediate oxaliplatin resistance in CRC

Interestingly, it has been demonstrated by some that tumor-suppressive miRNAs are associated with oxaliplatin resistance. Studies have identified that miR-1278 was significantly downregulated in CRC tissues, and that overexpression of miR-1278 inhibited CRC progression and enhanced oxaliplatin sensitivity through targeting of the KIF5B/BTG2 axis[59]. Similarly, miR-506 was weakly expressed in chemoresistant CRC cells, but its overexpression has been confirmed to impair the resistance of HCT116 cells to oxaliplatin *via* the Wnt/β-catenin pathway[60]. Moreover, miR-200b-3p was down-regulated in oxaliplatin-resistant CRC tissues and cells (HT29-OR and HCT116-OR), and studies revealed that miR-200b-3p reversed oxaliplatin resistance of CRC cells by targeting TUBB3[61]. Furthermore, miR-122, which was down-regulated in oxaliplatin-resistant SW480 and HT29 cells, has been demonstrated to be overexpressed in CRC cells, this could enhance the chemosensitivity of CRC by inhibiting the expression of XIAP[62]. As a tumor suppressor, miR-193a-5p can act directly upon CXCR4 to mitigate the chemosensitivity of CRC cells to 5-FU and oxaliplatin[63]. Using sequencing, Liang *et al*[64] discovered that miR-483-3p was negatively correlated with FAM171B expression, and targeting the miR-483-3p/FAM171B regulatory axis could enhance the sensitivity of CRC cells to oxaliplatin. Moreover, miR-325 mimics were observed to prevent the development of oxaliplatin resistance in CRC *via* interference with the HSPA12B/PI3K/AKT/Bcl-2 axis[65]. Finally, a proteomic analysis study reported that regulation of miR-195-5p and miR-497-5p is a potential strategy for alleviating oxaliplatin resistance in CRC cells[66].

As an additional factor, several groups showed that the interaction between ncRNAs and autophagy exerted significant roles in the therapeutic resistance of CRC[67]. Sun *et al*[68] demonstrated that miR-34a was expressed at low levels in oxaliplatin-resistant CRC patients and cells. Overexpression of miR-34a contributed to facilitating the sensitivity of CRC cells to oxaliplatin by regulating the TGF-β/Smad4 pathway resulting in the inhibition of macroautophagy. Furthermore, c-Myc has been shown to promote oxaliplatin resistance in CRC by integrating into the miR-27B promoter region and further mediating activation of the miR-27b-3p/ATG10 axis[69].

Table 1 Dysregulation of microRNAs and oxaliplatin resistance in colorectal cancer

MiRNAs	Expression[1]	Targets and pathways	Ref.
miR-135b-5p	↑	MUL1/ULK1	[49]
miR-454-3p	↑	PTEN	[50]
miR-19a	↑	PTEN/PI3K/AKT	[51]
miR-543	↑	PTEN/Akt/mTOR	[52]
miR-107	↑	CAB39/AMPK/mTOR	[53]
miR-503-5p	↑	PUMA	[54]
miR-744	↑	BIN1	[55]
miR-5000-3p	↑	USP49	[56]
miR-146b-5p	↑	WBSCR22	[57]
miR-19b-3p	↑	SMAD4	[58]
miR-1278	↓	-	[59]
miR-506	↓	Wnt/β-catenin	[60]
miR-200b-3p	↓	TUBB3	[61]
miR-122	↓	XIAP	[62]
miR-193a-5p	↓	CXCR4	[63]
miR-483-3p	↓	FAM171B	[64]
miR-325	↓	HSPA12B/PI3K/AKT/Bcl-2	[65]
miR-195-5p	↓	-	[66]
miR-497-5p	↓	-	
miR-34a	↓	TGF-β/Smad4	[68]
miR-27b-3p	↓	-	[69]

[1]Aberrant expression of miRNAs either up-regulated (↑) or down-regulated (↓) in CRC oxaliplatin resistance.
MiRNAs: MicroRNAs.

It is noteworthy that miR-181a could suppress the expression of BIRC6, a protein inhibitor of apoptosis, by directly targeting the 3'-UTR of BIRC6 mRNA[70]. Conversely, BIRC6 was observed to be significantly upregulated in acquired oxaliplatin-resistant CRC cells when compared to parental cells [71]. Considering that knockdown of BIRC6 helped enhance the chemosensitivity of CRC cells to oxaliplatin, targeting BIRC6 using miR-181a mimics may be a potential strategy to reverse oxaliplatin resistance in CRC.

Classification and biological functions of lncRNAs

LncRNAs are defined as a class of ncRNA molecules with transcript lengths of more than 200 nucleotides; they do not encode proteins due to their lack of an open reading frame[72]. LncRNAs can be classified into intergenic, intronic, sense, antisense, and bidirectional lncRNAs based on their genomic localization[73]. Alternatively, there are four categories of lncRNAs based on their functional mechanisms, including signal, decoy, guide, and scaffold lncRNAs[74,75]. It has been reported that lncRNAs can regulate transcription, translation, RNA stability, and alternative splicing[76-79]. It is noteworthy that numerous studies have revealed that aberrant lncRNAs (oncogenic and tumor suppressive lncRNAs) contribute to regulating the mechanism of oxaliplatin resistance through multiple cellular mechanisms, including but not limited to DNA damage repair, interference with drug influx and efflux, regulation of the cell cycle and apoptosis, and activation of signaling pathways[80,81]. Table 2 provides an overview of some aberrant lncRNAs and their target molecules in the context of oxaliplatin resistance in CRC.

Oncogenic lncRNAs as regulators of CRC oxaliplatin resistance

Studies discovered that high expression of the lncRNA GIHCG promoted the proliferation, migration, and invasion of tumor cells, and enhanced their resistance to 5-FU and oxaliplatin[82]. Similarly, overexpression of the lncRNA ARSR reduced cell apoptosis and induced oxaliplatin resistance[83]. Notably,

Table 2 Dysregulation of long non-coding RNAs and oxaliplatin resistance in colorectal cancer

LncRNAs	Expression[1]	Targets and pathways	Ref.
GIHCG	↑	-	[82]
lncARSR	↑	-	[83]
HOTAIR	↑	miR-1277-5p/ZEB1	[85]
MALAT1	↑	MALAT1/miR-218/EZH2	[86]
	↑	miR-324-3p/ADAM17	[88]
OIP5-AS1	↑	miR-137	[89]
Linc00152	↑	miR-193a-3p/ERBB4/AKT	[90]
CASC15	↑	miR-145/ABCC1	[92]
CBR3-AS1	↑	miR-145-5p	[93]
CRNDE	↑	miR-136/E2F1	[94]
LINC00460	↑	miR-149-5p/miR-150-5p/p53	[95]
KCNQ1OT1	↑	miR-34a/Atg4B	[96]
MIR155HG	↑	miR-650/Annexin A2	[91]
lnc-RP11-536 K7.3	↑	SOX2/HIF-1α/USP7	[97]
TUG1	↑	GATA6/BMP	[98]
LUCAT1	↑	UBA52/RPL40/MDM2/p53	[99]
PiHL	↑	EZH2/HMGA2/PI3K/Akt	[100]
ELFN1-AS1	↑	EZH2/DNMT3a/MEIS1	[102]
SNHG5	↑	STAU1	[103]
CCAT2	↑	BOP1/AURKA	[104]
NBAT-1	↓	WWC3/LATS1/YAP	[105]
MEG3	↓	-	[106]
		miR-141/PDCD4	[107]
lnc-AP	↓	pep-AP/TALDO1	[108]
lncRNA PVT1	-	hsa-miR-297/GSTA2	[109]

[1]Aberrant expression of lncRNAs either up-regulated (↑) or down-regulated (↓) in CRC oxaliplatin resistance.
LncRNAs: Long non-coding RNAs.

emerging evidence has revealed that lncRNAs could target mRNAs to promote chemoresistance[84]. For instance, upregulation of the lncRNA HOTAIR has been reported to facilitate EMT in a ZEB1-dependent manner by negatively regulating miR-1277-5p, a process that is involved in hypoxia-induced oxaliplatin resistance[85]. We previously discovered that metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was overexpressed in CRC tissues compared to paired noncancerous tissues. Correspondingly, the overexpression of MALAT1 suppressed E-cadherin expression and promoted oxaliplatin-induced EMT by interacting with EZH2. Furthermore, upregulated MALAT1 further inhibited miR-218 expression, resulting in poor response to oxaliplatin-based chemotherapy in CRC patients[86]. Numerous studies have demonstrated that some endogenous transcripts (such as endogenous pseudogenes, lncRNAs, and circRNAs) can complement miRNAs in sequences and inhibit their expression, resulting in the upregulation of target gene expression. This phenomenon is called the miRNA "sponge" effect[87]. Analogously, Fan *et al*[88] demonstrated that MALAT1 could function as a "sponge" for miR-324-3p and increase the expression of ADAM17 to facilitate resistance to oxaliplatin in CRC cells.

Moreover, lncRNA Opa-interacting protein 5 antisense RNA 1 can complement the sites of miR-137 in sequence, sponging miR-137 and inhibiting its expression, thus conferring oxaliplatin resistance in CRC cells[89]. It was reported that Linc00152 increased ERB-B2 receptor tyrosine kinase 4 expression by acting as a "sponge" for miR-193a-3p resulting in the promoted phosphorylation of AKT at Thr308 and Ser47; this process mediated oxaliplatin resistance in CRC cells[90]. In addition, researchers have illustrated that the lncRNA MIR155HG promoted M2 macrophage polarization and enhanced

oxaliplatin resistance in CRC cells by regulating the miR-650/Annexin A2 axis[91]. Similarly, other lncRNAs such as CASC15[92], CBR3-AS1[93], CRNDE[94], LINC00460[95], and KCNQ1OT1[96] have been reported to serve as "sponges" for miRNAs, thereby mediating oxaliplatin resistance in CRC.

Several research groups have revealed that lncRNAs could stabilize functional proteins, and mediate the chemoresistance of cancers. For example, lncRNA-RP11-536 K7.3 contributed to oxaliplatin resistance by recruiting SOX2 to activate deubiquitinase USP7 and stabilize the protein HIF-1α in CRC cells[97]. Furthermore, the lncRNA TUG1 induced oxaliplatin resistance in CRC stem cells and inhibited cell apoptosis by interacting with GATA6[98]. Moreover, two additional studies have further revealed that the lncRNAs LUCAT1 and PiHL mediated oxaliplatin resistance in CRC cells by interacting with UBA52 and EZH2 proteins, respectively[99,100].

LncRNAs have also been demonstrated to be regulators of gene expression by enhancing the stability of mRNA through epigenetic mechanisms[101]. Studies have elucidated that the lncRNA ELFN1-AS1 suppressed myeloid ecotype virus insertion site 1 transcription and promoted oxaliplatin resistance by interacting with EZH2 and DNA methyltransferase 3 alpha after localization to the promoter region of MEIS1 gene[102]. In addition, overexpression of the lncRNA SNHG 5 contributed to the proliferation of CRC cells and their resistance to oxaliplatin-induced apoptosis by blocking the degradation of SPATS 2 by STAU 1[103]. Of note, it has been identified that in microsatellite stable CRC, overexpression of the lncRNA CCAT2 could induce chromosomal instability, and enhance 5-FU and oxaliplatin-resistance by the upregulation of ribosomal biogenesis factor and the activation of aurora kinase A[104].

Tumor-suppressive lncRNAs as regulators of CRC oxaliplatin resistance

Previously, several tumor suppressor lncRNAs were reported to be associated with CRC oxaliplatin resistance. For instance, the expression levels of NBAT-1 and MEG3 in CRC tissues were low compared with normal adjacent tissues, especially in oxaliplatin-resistant patients[105,106]. Mechanistically, NBAT-1 was revealed to inhibit the growth of oxaliplatin-resistant CRC cells by activating the WWC 3/LATS 1/YAP pathway after acting as a "sponge" for miR-454[105]. Similarly, MEG3 promoted the sensitivity of CRC cells to oxaliplatin by regulating the miR-141/PDCD4 axis[107]. It was further reported that lnc-AP is a lncRNA with coding potential, and is highly associated with oxaliplatin resistance in CRC; lnc-AP encodes the short peptide pep-AP that enhances sensitization of CRC cells to oxaliplatin *via* the pep-AP/TALDO1 pathway[108]. Intriguingly, an epidemiological investigation demonstrated that an rs2278176 CT/TT mutation in the lncRNA PVT1 increased the chemosensitivity of CRC patients to FOLFOX compared to CRC patients carrying distinct genotypes[109].

Of note, when compared to parental cells, the lncRNA CRNDE was significantly down-regulated in oxaliplatin-resistant cells and can be considered a predictor of oxaliplatin treatment response and tumor prognosis[110]. Interestingly, this lncRNA can be combined with arginine-rich splicing factor 6 to reduce its stability, knockdown of the latter can inhibit autophagy and increase the sensitivity of cancer cells to oxaliplatin by the alternative splicing of PICALM[111]. Given that up-regulated expression of CRNDE aids an increase in the sensitivity of cancer cells to oxaliplatin, we speculate that increasing CRNDE expression and decreasing the stability of SRSF6 may be a promising strategy to ameliorate CRC resistance to oxaliplatin.

Classification and biological functions of circRNAs

Most circRNAs consist of covalently closed loop single-stranded RNAs produced by the back-splicing of exon precursor mRNAs that are more stable than their linear precursor gene[30]. Currently, circRNAs consist of four general categories: Exonic circRNAs, circular intronic RNAs, exon-intron circRNAs, and circRNA from other sources (such as antisense circular RNA or intergenic circular RNA) based on their structural domains and biogenesis features[112,113]. As vital biological regulators, circRNAs have been reported to widely participate in multiple oxaliplatin resistance-related mechanisms, such as apoptosis, autophagy, glycolysis, TME, EMT, DNA damage repair, *etc*[114]. Studies identified that hsa_circ_0040-809 was highly expressed in CRC cells and tissues; hsa_circ_0040809 upregulates DNMT1 expression by competitively combining with miR-515-5p, thus suppressing apoptosis and promoting cancer progression[115]. Conversely, as a tumor suppressor, circTADA2A was found to be down-regulated in cancer cells and tissues. Overexpression of circTADA2A elevated the expression of KLF14 by targeting miR-374a-3p, thereby promoting cell apoptosis and inhibiting tumor growth[116]. Moreover, has_circ_0001666 is a circRNA originating from exons 2, 3, and 4 of the host gene FAM120B. Studies have demonstrated that hsa_circ_0001666 competitively bound to miR-576-5p to increase PCDH10 expression, which in turn restrained cell stemness and EMT, and the Wnt/β-catenin pathway[117]. Zhang *et al*[118] revealed that the knockdown of circRNA_103948 contributed to enhanced autophagy of CRC cells by targeting the miR-1236-3p/TPT1 signaling pathway, inhibiting tumor growth. In addition, hypoxia facilitated circCCDC66 expression, which boosted the development of CRC by modulating the miR-3140/autophagy axis[119]. Furthermore, studies observed that hsa_circ_101555 was more highly expressed in CRC tissues than in normal tissues and was associated with adverse prognosis of patients. Inhibition of circ101555 resulted in the initiation of cell apoptosis and devastated DNA repair by activating the miR-597-5p/CDK6/RPA3 axis, thereby repressing the progression of CRC[120]. These studies suggested that circRNAs might be closely associated with the process leading to CRC oxaliplatin resistance.

CircRNAs regulate CRC oxaliplatin resistance

The majority of circRNAs containing miRNA response elements (MREs) can regulate gene expression and signaling pathways by functioning as ceRNAs, mediating tumor chemoresistance[121] (Table 3). Studies have identified that circular RNA protein tyrosine kinase 2 was significantly up-regulated in CRC tissues compared to normal tissues, and its high levels of expression promoted CRC progression and oxaliplatin resistance by regulation of the miR-136-5p/YTHDF1 axis[122]. Similarly, circ_0032833 was highly expressed in FOLFOX-resistant CRC cells, and knock-down of circ_0032833 could promote apoptosis and partially enhance the sensitivity of CRC cells to 5-FU and oxaliplatin through the activity of miR-125-5p/MSI1[123]. Lai *et al*[124] also demonstrated that hsa_circ_0079662 functioned as a "sponge" for hsa-mir-324-5p and activated HOXA9 through activation of the TNF-α pathway; this process induced oxaliplatin-resistance in CRC cells. Furthermore, our previous research revealed that circHIPK3 was highly expressed in chemoresistance CRC patients, and this circRNA was strongly associated with tumor size, regional lymph node metastasis, and distant metastasis. The following functional investigation revealed that circHIPK3 facilitated oxaliplatin resistance, but not 5-FU resistance, by restraining autophagy-related cell death. Mechanistically, circHIPK3 acted as a ceRNA and a "sponge" for miR-637 resulting in activation of the STAT3/Bcl-2/Beclin1 pathway, and subsequent induction of oxaliplatin-resistance in CRC cells[125].

Intriguingly, oxaliplatin could directly induce the overexpression of some oncogenes, further exacerbating chemoresistance. Circular CCDC66, derived from exons 6 to 11, was highly expressed in oxaliplatin-resistant CRC tissues and cells, a phenomenon caused by oxaliplatin-triggered cell stress through phosphoinositide 3-kinase related kinases-mediated phosphorylation of DHX9[126]. Despite extensive literature, many circRNAs remain to be explored in the study of oxaliplatin-resistant CRC. Abu *et al*[127] identified differential expression of circular RNAs when analyzing chemosensitive and chemoresistant CRC cells using microarray; hsa_circ_32883 and hsa_circ_0338 were screened as promising candidates. Subsequently, their study also identified that exosome-derived circRNAs (hsa_circ_0032883, hsa_circ_0002039, and hsa_circ_0000338) may play important roles in the manifestation of chemoresistance[128].

Studies have reported that the expression of exosomal miR-21-5p from oxaliplatin-resistant cells was significantly elevated, and therefore levels of this miRNA could act as a predictor of chemotherapy response in CRC patients[129]. Interestingly, miR-21-5p can be sponged by some tumor-suppressive circRNAs, such as circDDX17[35] and circEPB41L2[130], thus resulting in activation of the downstream PTEN/AKT pathway. Importantly, previous studies have confirmed that targeting the PTEN/PI3K/AKT/mTOR pathway partially reversed oxaliplatin resistance in CRC[51,52]. Given this, we speculate that targeting these suppressive circRNAs might contribute to the sensitization of CRC cells to oxaliplatin *via* the miR-21-5p/PTEN/AKT axis. Furthermore, hsa_circ_0001955 and hsa_circ_0000977, potential upstream targets for chemoresistance-associated miRNAs[49,93], were demonstrated to be dysfunctional in CRC tissue, a finding that suggests these circRNAs may be involved in the progression of CRC chemoresistance in a ceRNA-dependent manner[131]. Currently, studies regarding the roles of circRNAs in oxaliplatin resistance are less commonly reported, the precise and comprehensive mechanisms of circRNAs in CRC chemoresistance need to be explored by further study.

INTEGRATION, APPLICATION, AND CHALLENGES OF TARGETING EXOSOMAL NCRNAS IN CRC CHEMORESISTANCE

Exosomes biogenesis and characterization

Extracellular vesicles (EVs) are defined as a class of membranous vesicles that are released by cells to the extracellular matrix and play a key role in various physiological and pathological processes. According to the difference in their origin, size, content, and biological function, EVs can be roughly divided into three main subtypes—microbubbles, exosomes, and apoptotic bodies[132]. Exosomes are an emerging hallmark of liquid biopsy and have attracted much scientific attention of late, these small disc-shaped EVs have a diameter of approximately 40–150 nm and are enclosed by a lipid bilayer membrane[133]. Almost all cell types can secrete exosomes, and they also exist broadly within fluids including blood, urine, saliva, milk, ascites, cerebrospinal fluid, and others[40,134]. At present, ultracentrifugation remains the standard method for exosome extraction. However, with the emergence of novel technologies, exosome isolation and purification strategies are being continuously optimized, such as size exclusion chromatography, magnetic bead immune capture, and microfluidic-based chip technology techniques[135], these are aimed at efficiently obtaining exosomes with high abundance and quality. The characterization of exosomes has emerged as a prerequisite for their utilization, and the three basic characterization modes of morphological identification are particle size distribution and protein markers. However, more advanced features are gradually being included, in exosome characterization, such as the identification of the purity of exosomes and their uptake[135,136]. As signaling entities, numerous studies have demonstrated that exosomes exert biological effects in two ways: Firstly,

Table 3 Dysregulation of circular RNAs and oxaliplatin resistance in colorectal cancer

CircRNAs	Expression[1]	Targets and pathways	Ref.
circPTK2 (hsa_circ_0003221)	↑	miR-136-5P/YTHDF1	[122]
circ_0032833	↑	miR-125-5p/MSI1	[123]
hsa_circ_0079662	↑	hsa-mir-324-5p/TNF-α/HOXA9	[124]
circHIPK3	↑	miR-637/STAT3/Bcl-2/Beclin1	[125]
Circular CCDC66	↑	PI3KK/p-DHX9	[126]
hsa_circ_32883/ hsa_circ_0338	↑	-	[127,128]

[1]Aberrant expression of circRNAs either up-regulated (↑) or down-regulated (↓) in CRC oxaliplatin resistance.
CircRNAs: Circular RNAs.

exosomal membrane proteins or lipids act as ligands, directly activating receptors at the surface of target cells, generating cascade signal events and activating intracellular signaling pathways; Secondly, exosomes entrain and transport cellular signal-regulating molecules to target cells; these include DNA, lipids, proteins, and RNAs, which are extensively involved in tumorigenesis, metastasis, recurrence, and chemoresistance of cancers[137,138]. In particular, exosomal ncRNAs have received unprecedented appreciation in biomedical research and clinical application recently[139,140]. Several exosomal ncRNAs involved in CRC oxaliplatin resistance are listed in Table 4.

The latent mechanisms of exosomal ncRNAs in CRC oxaliplatin resistance

Increasing numbers of reports have revealed that exosomal ncRNAs can mediate chemoresistance *via* remodeling of the tumor microenvironment. Studies have identified that lncRNA H19 is highly expressed in CRC tissues compared to normal tissues. Moreover, lncRNA H19 derived from CAFs can act as a direct "sponge" for miR-141 and activate the β-catenin pathway, thus promoting stemness and oxaliplatin resistance[141]. Similarly, exosomal miR-92a-3p was transmitted from CAFs to cancer cells, promoting chemoresistance *via* the Wnt/β-catenin/apoptosis pathway[20]. Moreover, CAF-derived exosomes were revealed to deliver CRC-associated lncRNA to cancer cells, interacting with the mRNA of the stable protein human antigen R and increasing the expression of β-catenin; these processes promoted oxaliplatin resistance in CRC cells[142]. Qu *et al*[143] also found that exosomal cricN4BP2L2 secreted by CAFs can interact with EIF4A3 and modulate the PI3K/AKT/mTOR signaling pathway, thus promoting oxaliplatin resistance and the stemness of CRC cells. In addition, exosomal circ_0094343 derived from human colonic epithelial cells (NCM460 cells) was identified to be taken up by CRC cells in which it regulated cell apoptosis and glycolysis *via* the miR-766-5p/TRIM67 pathway, thereby enhancing the chemosensitivity of tumor cells[144].

Conversely, tumor cells have been revealed to generate exosomes in an autocrine or paracrine manner; they are taken up by themselves or delivered to the local microenvironment, thus exerting functional regulation. Ning *et al*[145] demonstrated that CRC cell-derived exosomal miR-208b could promote Treg expansion by directly inhibiting the expression of programmed cell death factor 4, resulting in promoted tumor progression and oxaliplatin resistance. Similarly, studies have revealed that exosomal miR-46146 conferred chemoresistance *via* the targeting of programmed cell death factor 10 in CRC cells[146]. Interestingly, one particular study revealed that miR-1915-3p was down-regulated in oxaliplatin-resistant CRC cells compared to oxaliplatin- sensitive cells and that EVs-derived miR-1915-3p increased the oxaliplatin sensitivity of drug-resistant cells by targeting PFKFB3/USP2[147]. Moreover, it has been reported that hsa_circ_0005963 can be transferred from oxaliplatin-resistant cells to sensitive cells by exosomes, directly acting as a miR-122 "sponge" and increasing the expression of PKM2, which promotes glycolysis and oxaliplatin-resistance in sensitive cells[148].

Exosomal ncRNAs as potential liquid biopsy biomarkers

Liquid biopsy, in which diseases are evaluated *via* sampling biological fluids, can avoid the influence of tissue heterogeneity on tumor molecular typing to some extent. More and more evidence show that abundant exosomes are enriched in body fluids and participate in many physiological and pathological processes. As an emerging hallmark of liquid biopsy, exosomes are of substantial value in cancer diagnosis, monitoring, and prediction[136,140]. Studies found that the ratio of exosomal miRNAs is an effective predictor of tumor response in peritoneal metastatic patients after repeated intraperitoneal chemotherapy, as patients with high ratios of miR-223-3p/miR-29b-3p or miR-21-5p/miR-29b-3p had inferior survival outcomes compared to patients with low ratios[149]. By analogy, exosomes derived

Table 4 Aberrant exosomal non-coding RNAs and their targets in the chemoresistance of colorectal cancer

Exosomal ncRNAs[1]	Donor cells	Recipient cells	Targets and pathways	Ref.
miR-21↓	THLG-293T/LG-293T cells	CRC cells	-	[156]
miR-208b↑	CRC cells	T cells	PDCD4	[145]
miR-92a-3p↑	CAFs cells	CRC cells	FBXW7/MOAP1	[20]
miR-46146↑	CRC-R cells	CRC-S cells	PDCD10	[146]
miR-1915-3p↓	FHC cells	CRC cells	PFKFB3/USP2	[147]
lncRNA H19↑	CAFs cells	CRC cells	-	[141]
lncRNA CCAL↑	CAFs cells	CRC cells	HuR/β-catenin	[142]
cricN4BP2L2	CAFs cells	CRC cells	EIF4A3/PI3K/AKT/mTOR	[143]
ciRS-122↑	CRC-R cells	CRC-S cells	miR-122/PKM2	[148]
circ_0094343↓	NCM460 cells	CRC cells	miR-766-5p/TRIM67	[144]

[1]Aberrant exosomal ncRNAs are either up-regulated (↑) or down-regulated (↓) in CRC oxaliplatin resistance. CRC-R cells and CRC-S cells represent oxaliplatin-resistant and oxaliplatin-sensitive cells, respectively.
ncRNAs: Non-coding RNAs; CRC: Colorectal cancer; CAFs: Cancer-associated fibroblasts.

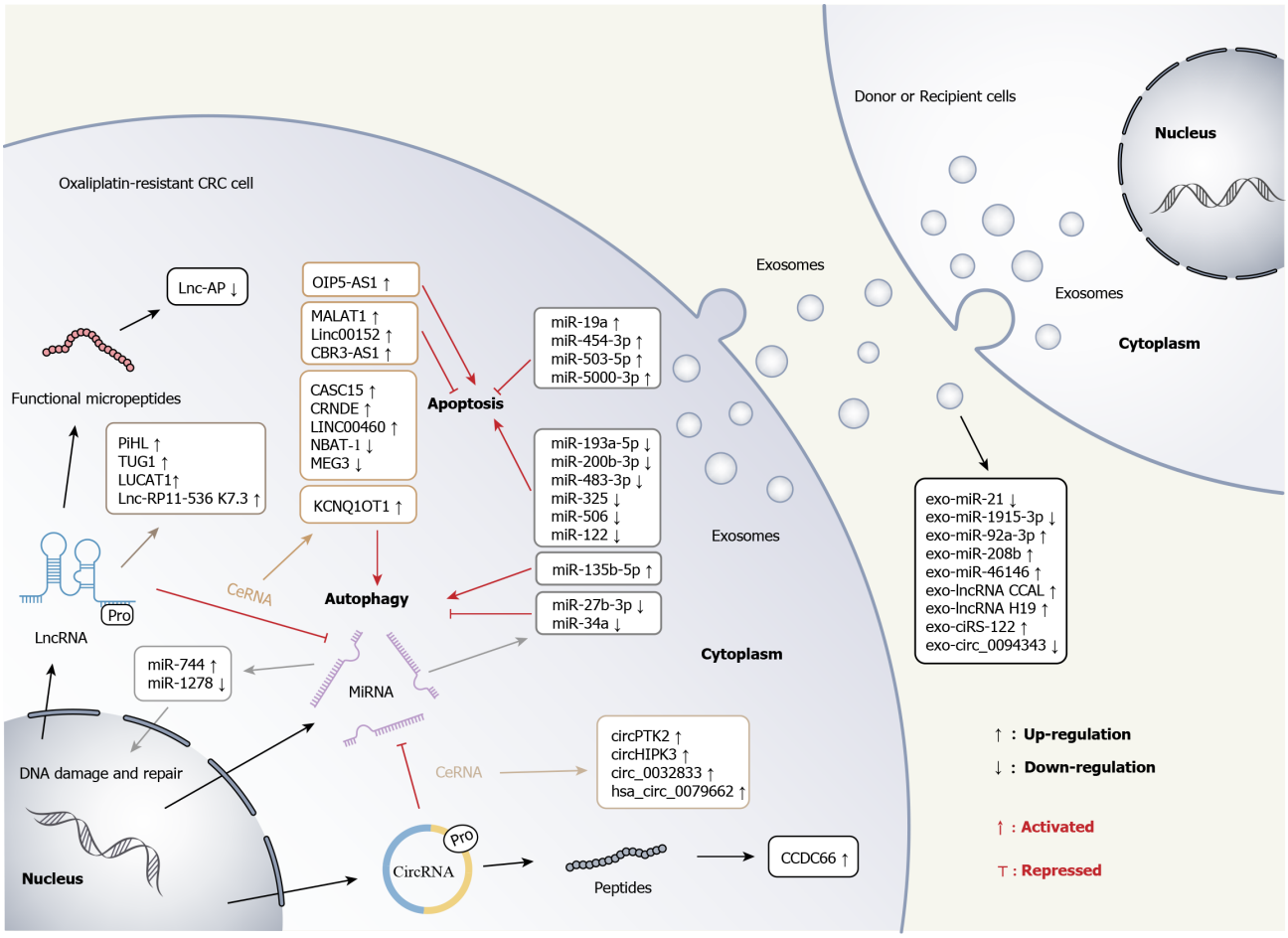
miR-21-5p, miR-1246, miR-1229-5p, and miR-96-5p were highly expressed in CRC patients and cancer cell lines resistant to 5-FU and oxaliplatin compared with those sensitive to chemotherapy. The area under the curve (AUC) of four combinations of exosomal miRNAs was 0.804, which is expected to be an effective predictor of chemotherapy[129]. In addition, researchers found that plasma-derived exosomal miRNA-125b was highly expressed in patients with progressive disease (PD) compared with the healthy control group or patients with stable disease. Importantly, some patients with PD who respond to modified fluorouracil, leucovorin, and oxaliplatin (mFOLFOX6) have obvious differences in the expression of plasma exosomal miRNA-125b before and after chemotherapy, and patients with low expression of exosomal miRNA-125b have higher progression-free survival (PFS)[150]. Furthermore, it was reported that miR-208b was highly expressed in the serum of FOLFOX-resistant CRC patients and the AUC of serum miRNA-208b was 0.771 [95% confidence interval (CI): 0.688-0.855], which is better than that of serum CEA of 0.493 (95%CI: 0.385-0.601). Their research suggests that this miRNA can be regarded as a promising liquid biopsy indicator to predict FOLFOX sensitivity in cancer patients[145]. Encouragingly, a panel of plasma exosomal miR-17-5p and miR-185-5p were successfully used to predict FOLFOX4/FOLFIRI responses in patients with advanced CRC[151].

Exosomal ncRNAs as delivery vehicles for chemosensitization

A large number of reports have revealed the prospects for the application of exosomes to overcome chemoresistance, their strong stability, intrinsic biocompatibility, low immunogenicity, and natural targeting ability making them favorable targets[152,153]. Novel research has exhibited an engineered exosome encapsulating oxaliplatin and PGM5 Antisense RNA 1 that were delivered into CRC cells, and these effectively relieved chemoresistance and inhibited tumor progression[154]. Furthermore, Pi *et al* [155] constructed engineered EVs enwrapped in folate nanoparticles and survivin siRNA, which significantly inhibited tumor growth in CRC xenograft mice. Similarly, miR-21 inhibitors have been co-incorporated into exosomes, resulting in enhanced chemosensitivity of CRC cells[156]. Taken together, these findings suggest that exogenous delivery of ncRNAs could serve as a novel therapeutic target for CRC.

CONCLUSION

A multitude of emerging studies have confirmed that dysfunctional ncRNAs contribute to the development of malignancies, including chemoresistance. In this review, we systematically summarized the multiple mechanisms by which ncRNAs function in CRC oxaliplatin resistance and discussed the biomedical prospects of exosomal ncRNAs as hallmarks for fluid biopsy and therapeutic targets. Figure 2 schematically illustrates the multiple regulatory roles of ncRNAs in CRC oxaliplatin resistance. Owing to the differential expression patterns of ncRNAs in cancer patients, it is considered they may be used as biomarkers for early detection, tumor staging, and clinical outcome. Although many mechanisms by which ncRNAs exert their biological effects remain to be deciphered, targeting these ncRNAs has emerged as a promising strategy to ameliorate chemoresistance.



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Figure 2 Overview of the mechanisms by which several non-coding RNAs exert oxaliplatin resistance in colorectal cancer. Various microRNAs, long non-coding RNAs, and circular RNAs are up-regulated or down-regulated in colorectal cancer and mediate oxaliplatin resistance *via* a multitude of mechanisms. These mechanisms include acting as competing endogenous RNAs, generating encoded peptides, regulating autophagy, inducing apoptosis, and interfering with DNA damage and repair. In particular, several non-coding RNAs have been demonstrated to confer oxaliplatin resistance in colorectal cancer cells *via* exosomes. CRC: Colorectal cancer; lncRNAs: Long non-coding RNAs; miRNAs; MicroRNAs; circRNAs: Circular RNAs.

Nevertheless, the integration and application of ncRNAs in clinical practice are far from the existing research fervor and are currently being tested in experimental studies; there are still many challenges to be overcome before ncRNAs can be extensively implemented in clinics. Firstly, the results of existing research are confounding due to differing nomenclature being utilized for circRNAs. The circRNAs labeled using different naming standards make the subsequent genomic localization cumbersome. Secondly, the expression of ncRNAs in biological fluids is relatively low, and therefore accurate detection and quantification of these molecules is a prerequisite for the development of biomarkers. Furthermore, due to differences in the lengths and mechanisms by which ncRNAs function in distinct tumor types, it is a challenge to select appropriate targets for these multitudinous candidates. Exosomes may be a better intermediary for delivering ncRNAs therapies; this could be achieved in an endogenous or exogenous manner. However, it is crucial to increase the loading of ncRNAs delivered by endogenous exosomes and ensure the safety of engineered exosomes with complex structures. Measurements of the efficacy of exosomal ncRNAs in clinical applications cannot be limited to laboratory data alone. A series of large validation cohorts will be required to obtain reliable predictive models and accurate therapeutic doses. In the future, clinical translational trials utilizing target ncRNAs are expected to overcome oxaliplatin resistance and thus improve the clinical outcomes of CRC patients.

FOOTNOTES

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Country/Territory of origin: China

ORCID number: Zheng-Dong Luo 0000-0002-4304-2695; Yi-Feng Wang 0000-0001-5955-2449; Yu-Xiao Zhao 0000-0003-0503-8633; Long-Chen Yu 0000-0001-6101-3751; Tian Li 0000-0001-7061-6925; Ying-Jing Fan 0000-0002-3457-8121; Shun-Jie Zeng 0000-0002-5975-3280; Yan-Li Zhang 0000-0002-3424-9731; Yi Zhang 0000-0002-0440-1798; Xin Zhang 0000-0003-2138-5600.

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REVIEW

Microbiota of the gastrointestinal tract: Friend or foe?

Marina A Senchukova

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Marina A Senchukova, Department of Oncology, Orenburg State Medical University, Orenburg 460000, Russia

Corresponding author: Marina A Senchukova, MD, PhD, Professor, Department of Oncology, Orenburg State Medical University, Sovetskaya Street, Orenburg 460000, Russia.
masenchukova@yandex.com

Abstract

The gut microbiota is currently considered an external organ of the human body that provides important mechanisms of metabolic regulation and protection. The gut microbiota encodes over 3 million genes, which is approximately 150 times more than the total number of genes present in the human genome. Changes in the qualitative and quantitative composition of the microbiome lead to disruption in the synthesis of key bacterial metabolites, changes in intestinal barrier function, and inflammation and can cause the development of a wide variety of diseases, such as diabetes, obesity, gastrointestinal disorders, cardiovascular issues, neurological disorders and oncological concerns. In this review, I consider issues related to the role of the microbiome in the regulation of intestinal barrier function, its influence on physiological and pathological processes occurring in the body, and potential new therapeutic strategies aimed at restoring the gut microbiome. Herewith, it is important to understand that the gut microbiota and human body should be considered as a single biological system, where change of one element will inevitably affect its other components. Thus, the study of the impact of the intestinal microbiota on health should be considered only taking into account numerous factors, the role of which has not yet been fully elucidated.

Key Words: Gut microbiota; Bacterial metabolites; Intestinal barrier; Dysbiosis; Fecal microbiota transplantation; Probiotics

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Core Tip: The gut microbiota affects the development and functioning of all body systems, providing metabolic, physiological, regulatory and protective functions. Violations in the qualitative and quantitative composition of the microbiome lead to the development of a wide variety of diseases, such as diabetes, obesity, cardiovascular issues, neurological disorders and oncological concerns. Considering that intestinal dysbiosis plays a key role in the development of a number of diseases, aim to normalize the microbiome seems to be a greatly perspective direction in their prevention and treatment.

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INTRODUCTION

Trillions of microorganisms, known as microbiota, colonize the human body. The human gastrointestinal tract harbours more than 1000 species of bacteria belonging to a relatively few known bacterial phyla[1]. Features of their mutual coexistence determine the nature of various physiological and pathological processes occurring in the human body. To discuss these issues, a search was made in the PubMed database (https://pubmed.ncbi.nlm.nih.gov/) and *Reference Citation Analysis* (https://www.referencecitationanalysis.com/) for studies published up to July 1, 2022 using a combination of text keywords "gut microbiota", "bacterial metabolites", "intestinal barrier", "dysbiosis", "fecal microbiota transplantation", and "probiotics". A total of 846 unique results were identified, which were screened individually by title and abstract and were included based on the role of the microbiome in the regulation of intestinal barrier function, its influence on physiological and pathological processes occurring in the body, and new therapeutic strategies aimed at restoring the gut microbiome.

It is believed that bacteria begin to colonize the human intestine immediately after birth and, possibly, even in utero[2,3]. Breast milk plays a crucial role in the composition of the microbial community within newborns through transfer of the milk microbiota to the infant's gut[4]. In the first 6 mo of a child's life, there is a steady increase in the number of *Enterobacteriaceae*, *Bifidobacteriaceae* and *Clostridiaceae*. However, the microbiome of children differs depending on diet, gender, race and ethnicity[5,6]. In addition, the mode of delivery may affect the composition of the gut microbiota of early infants[3,7]. In particular, in children born by cesarean section, there is a high abundance of *Bifidobacterium*, and *Clostridium* genera and the family Enterobacteriaceae, along with a low abundance of *Streptococcus* and *Ruminococcus* genera[8]. Moreover, in children born by cesarean section, the *Bacteroides* genus is not detected in the feces until 6-18 mo after birth[9].

Microorganisms living in the gut of adults include bacteria, fungi, protozoa, archaea, and some viruses[10]. The total number of bacteria in a 70 kg "reference man" is estimated at 3.8×10^{13} cells, which is comparable to the number of human host cells (3.0×10^{13})[11]. The specific microbiota at the genus and species levels varies depending on geography, environment, diet, age, genotype, presence of diseases and lifestyle[12,13]. For instance, if the prevalence of proteins and animal fats in the diet exists, *Bacteroides* will predominate in the microbiota, and if there is a high level of carbohydrates, then *Prevotella* will ascendant. The gut microbiota encodes over 3 million genes, which is around 150 times more than the number of genes in the host genome[14]. Approximately 90% of the composition of the gut microbiome is represented by *Firmicutes* (79.4%), *Bacteroidetes* (16.9%), *Actinobacteria* (2.5%) and *Proteobacteria* (1%)[5,15]. The number of microorganisms increases from the proximal to the distal gastrointestinal tract and from the epithelial layer to the lumen. This difference can be explained by the presence of a more aggressive environment in the upper intestines due to the incoming gastric acid, action of digestive enzymes, rapid movement of chyme and the decrease in partial pressure of oxygen in the distal gastrointestinal tract. For this reason, aerobic bacteria predominate in the small intestine, while facultative and obligate anaerobes predominate in the lower gastrointestinal tract. Furthermore, the distribution and organization of the gut microbiota is determined by intestinal mucins, which protect intestinal epithelial cells (IECs) from bacterial colonization. At the same time, the presence of the gut microbiota is a necessary condition for normal functioning of the mucosal barrier[16]. For example, mice treated with antibiotics had a thinner layer of mucus[17,18].

GUT MICROBIOTA AND INTESTINAL BARRIER

Partition of the body's internal environment from the intestinal microbiota is carried out by three types of barriers: physical, chemical and immunological. The physical barrier consists of epithelial cells, glycocalyx and a layer of mucus covering the surface of the gastrointestinal wall[19].

Physical barrier

The intestinal epithelium is the most rapidly self-renewing tissue in adult mammals. To maintain epithelial layer integrity, IECs are continuously replaced by proliferating progenitor cells derived from multipotent intestinal stem cells (ISCs) localized in the base of the crypts of Lieberkühn and the colon [20]. IECs differ in their proliferation ability, renewal rate and age. Aged IECs undergo apoptosis and are later ejected into the intestinal lumen, whereas Paneth cells leave the crypt bottom by cellular fragmentation and phagocytosis by macrophages infiltrating the lamina propria mucosae[21]. Under homeostatic conditions, the entire ileal crypt is replaced every 4-5 d[22].

It is customary to distinguish the following populations of the intestinal epithelium[22-24].

Columnar cells: Columnar cells (colonocytes) are the most numerous population of enterocytes. They participate in digestion due to the secretion of digestive enzymes, the absorption of digested products and transcellular transfer of dissociated monomers into the blood and lymph and take part in the exchange of bile acids and humoral immune response. Absorbent enterocytes produce polymeric immunoglobulin (Ig) receptor, which mediates transcytosis of dimeric IgA and polymeric IgM from the lamina propria through the epithelial barrier to the mucosal surface, ensuring the binding of bacteria and viruses on their surface and thereby preventing the penetration of pathogens into the internal environment of the body. In addition, during transcytosis of IgA through the epithelium, it can neutralize viruses that have entered cells, as well as bind and excrete proteins and other immune complexes on the surface of mucous[20,25].

Goblet cells: Goblet cells are a source of mucus. In addition, they can deliver small soluble antigens to dendritic cells (DCs) localized in the lamina propria and thus participate in the formation of immune tolerance to food antigens and the gut microbiome[20,26].

Enteroendocrine cells: Enteroendocrine cells secrete peptides and hormones (cholecystokinin, serotonin) to stimulate intestinal motility.

Tuft cells: Tuft cells participate in the clearance of parasites from the intestinal lumen due to the synthesis of interleukin (IL)-25, which is the key activator of type 2 innate lymphoid cells.

Paneth cells: Paneth cells in the small intestine or deep crypt secretory cells in the large intestine are the main regulators of microbial density in the intestines. When interacting with gram-negative bacteria, gram-positive bacteria or their products (lipopolysaccharides, lipoteichoic acids, lipid A, muramyl peptide), they secrete antimicrobial peptides (AMPs)[20,27,28]. Moreover, Paneth cells secrete important factors, such as epidermal growth factor, transforming growth factor-α, and Wnt ligands involved in stem cell maintenance[29].

Microfold cells: Microfold cells (M) are located in the follicle-associated epithelium overlying Peyer's patches and stimulate an immune response by binding luminal antigens for their further transport to subepithelial regions, where they are captured by DCs migrating to the mesenteric lymph nodes and stimulating the immune response[20]. The interaction of DCs with T cells stimulates an antigen-specific immune response directed against the pathogen or, conversely, leads to the induction of tolerance. Activation of B cells leads to the secretion of IgA, which plays an important role in the regulation of the gut microbiome. Immunoglobulin A, on the one hand, is involved in the binding and elimination of pathogenic bacteria, and on the other hand, it facilitates the translocation of commensals into Peyer's patches, activating the mechanisms of immunological tolerance and thereby stimulating the growth of intestinal symbionts[30,31].

It is worth noting that at the level of IECs, a structure composed of three junctions is formed: tight junctions (TJs), adherens junctions and desmosomes[20,32-34]. They provide mechanical strength between cells, intercellular adhesion and polarization and are also involved in cell signaling pathways [19]. The strength of the mechanical barrier also depends on the regeneration rate of the intestinal epithelium. It was established that the presence of intestinal microorganisms affects the number of Paneth cells and hence the integrity of the epithelial barrier, as Paneth cells regulate stem cell homeostasis[35]. Bacteria can directly damage TJ proteins or interfere with their synthesis *via* type 3 or type 4 secretion systems. The disruption of cell contacts can also be mediated by some bacterial enzymes and toxins, such as hemagglutinin/protease and ZO toxin, as well as bacterial metabolites, such as ethanol and acetaldehyde[13,36]. Apart from that, the gut microbiota can alter the mitochondrial metabolism of epithelial and immune cells, thereby activating inflammation and disrupting epithelial barrier function[37]. Integrity violation of the intestinal epithelium, the mucus barrier and cell contacts leads to the translocation of bacteria[38].

Recognition of the bacterial microbiota is carried out by TLR and NOD receptors, which are expressed by most IECs, including stem cells, enterocytes, goblet cells, enteroendocrine cells, Paneth cells, and M cells[19]. Most TLRs (TLR2, TLR3, TLR4, TLR5, and TLR9) are present on the basolateral membrane, while TLR2, TLR3, and TLR9 are also expressed on the apical surface. TLR5 expression is limited to Paneth cells in the epithelium of the small intestine and proximal colon[39,40]. Activation of innate immune system receptors, including TLR and NOD receptors, as well as inflammasomes, leads to

a signaling cascade that triggers the secretion of cytokines and chemokines, including IL-1β, IL-6, IL-12, IL-18, tumor necrosis factor alpha (TNF-α), CXCL8 and CCL20, which activate immunocompetent cells localized in the lamina propria[41-43]. Mitochondrial reactive oxygen species (mtROS), produced by immune cells, play a key role in the eradication of invading pathogens through direct bactericidal action or indirect impact on the activation of the NLRP3 inflammasome and the production of proinflammatory cytokines. Invading bacteria, as well as gut microbiome fermentation products such as short-chain fatty acids (SCFAs), induce mtROS production in immune cells through increased mitochondrial respiration and increased oxidative phosphorylation[44]. Hypoxia inducible factor-1α is believed to be the main regulator of mitochondrial responses during bacterial infection[37]. At the same time, damage to the intestinal epithelium mitochondria by toxins of pathogenic bacteria leads to the accumulation of mtROS and disruption of the barrier function of the epithelium[45,46].

Intestinal mucus (mucins)

Intestinal mucus is the first barrier for microorganisms in the gastrointestinal tract. It regulates nutrient and drug delivery, regulates symbiosis with the gut microbiota and protects the epithelium from dietary antigens and food toxins[20,47,48]. The thickness of the mucus layer in the small and large intestine is not the same. The main function of the small intestine is food digestion and absorption of nutrients, so the small intestine has a loose, discontinuous layer of mucus that can be easily removed. In the large intestine, where the density of microorganisms is much higher than in the small intestine, the number of mucus-producing goblet cells is significantly larger[20]. In the large intestine, the mucus layer is organized into two layers: an inner, dense, microbiota-free mucus layer and an outer layer, which is friable and permeable for microorganisms[13,17,31,49,50].

More than 20 mucin subtypes have been identified in humans. The best known and most studied one, found in the small and large intestines, is MUC2[17,49]. MUC2 is a highly O-glycosylated gelling mucin that forms polymeric networks *via* C-terminal dimerization and N-terminal trimerization. MUC2 monomers are glycosylated in proline, threonine, and serine-rich domains[51,52].

Mucus is secreted by goblet cells, grows rapidly and forms a stratified, dense layer that adheres to the epithelium[53]. On the one hand, mucus is necessary for the normal functioning of the gut microbiome, but on the other hand, the presence of the microbiome is a necessary condition for the normal functioning of the mucus barrier[54]. As noted above, mice treated with antibiotics have a thinner layer of mucus[17,18].

The barrier function of the mucus is confirmed by the fact that mice genetically deficient in Muc2 (Muc2 -/-) have bacteria invading the normally sterile distal colon crypts, which results in the development of spontaneous colitis[55], adenomas arising in the small intestine and an invasive cancer [56]. However, intestinal bacteria can directly influence the production and quality of intestinal mucus and hence the intestinal barrier permeability[57]. Bacteria and their metabolites that enter crypts are endocytosed by specialized goblet cells known as 'sentinel' goblet cells. This leads to the activation of TLR2/1, TLR4, and TLR5 Ligands with activated ROS synthesis, triggering the formation of the NLRP6 inflammasome and Ca2+-dependent compound exocytosis of MUC2-containing granules[58,59]. Importantly, increased regulatory secretion leads to the secretion of large amounts of MUC2 and the physical removal of bacteria from the crypt opening, thereby protecting the inferior crypt and multipotent ISCs, located at the bottom of the crypts, from bacterial invasion[20,60].

Chemical barrier

An important function of the chemical barrier is to maintain the abundance and composition of the gut microbiome. The chemical barrier includes AMPs, gastric acid, digestive enzymes, mucopolysaccharides, glycoproteins, glycolipids, and other compounds[19,61,62]. In addition, the composition of the microbiota can be influenced by various factors, such as hygiene, diet (especially the "Western diet" low in fiber and high in sugar and fat), oxygen concentration, microbial adhesion, host stress and other factors[17,49,63,64]. It is believed that microbiome regulation in the small intestine is mainly carried out by antibacterial peptides, while in the large intestine, this regulation is carried out through pattern recognition receptors[5]. The microbiome population is maintained, either by preventing colonization or through direct killing mechanisms.

The production of antimicrobials that lyse target cells is one of the main mechanisms for regulating the homeostasis of the gut microbiome. The contact of bacteria with Toll (TLR2, TLR4, TLR7 and TLR9), NOD1 and NOD2 receptors activates adapter proteins (for example, MyD88) and genes responsible for the synthesis of cytokines and chemokines in IECs[65-68]. In turn, the synthesis of cytokines by immune cells activates the genes responsible for the synthesis of AMPs[69]. Thus, mice deficient in MyD88 exhibit a 100-fold higher bacterial load in the gut than wild-type mice, and this increase is correlated with a decrease in the antibacterial peptide RegIIIgγ[70]. Paneth cells expressing TLR5 are major producers of antimicrobials, many of which are cationic AMPs that interact with negatively charged bacterial membranes and destroy them[12]. Interestingly, TLR5 expression occurs predominantly in intestinal crypts and is genetically determined, as TLR5 expression does not require bacterial or immune signals. It is believed that their main function may be related to the protection of Paneth cells and stem cells[31,71].

Other AMPs are also involved in the regulation of the gut microbiome population. For example, protein 8 (Lypd8) is highly expressed by colonocytes and facilitates segregation of microorganisms in the colon *via* flagella binding[61]. The lectin-like protein ZG16 specifically binds the peptidoglycan of gram-positive bacteria and thereby inhibits their penetration into the inner layer of the colon[62]. Lectins RegIIIγ from IECs and beta-defensins from neutrophils have a bactericidal effect against a number of bacteria. However, *Firmicutes* and *Bacteroidetes* living in the small intestine are resistant to these antibacterial agents[72]. It is also worth noting that some bacteria can synthesize bacteriocins (for example, colicin and microcins), which inhibit the growth of competitors[73,74].

Immunological barrier

The gut immune barrier is represented by single lymphoid follicles and Peyer's patches — peripheral accumulations of lymphoid cells located in the lamina propria of the small intestine mucosa[20]. Within the follicles, there are various immune elements, including B and T lymphocytes, DCs, and neutrophils, that secrete cytokines and antibodies in response to antigen entry. Goblet cells are involved in the presentation of luminal antigens to the CD103+ DC complex of the intestinal mucosa lamina propria, forming antigenic complexes (goblet cell-associated antigen passages)[75]. Secretory IgA (SIgA), another component of the intestinal barrier, is produced by plasma cells (50 mg/kg SIgA daily in an adult) and localized predominantly in the lamina propria of the intestinal mucosa[31]. It is believed that SIgA is able to interact with commensal intestinal bacteria, mediating the formation of a bacterial biofilm. SIgA is resistant to the action of intestinal proteases, which provide protection for bacteria. SIgA can penetrate through the epithelial lining into the intestinal lumen, bind antigens and deliver them to the immune cells of the lymphoid tissue[76].

Elements of immune protection can also include an increase in "tolerance" to a microbe (or toxin of microbial origin)[52] and the death of infected cells. In particular, flagellins of pathogenic bacteria that have overcome the epithelial barrier are able to activate NAIP/NLRC4 in macrophages, which causes the death of infected epithelial cells and their expulsion into the intestinal lumen[77].

It is important to note that the intestine is the most important immune organ, which not only protects against external pathogens but also participates in the formation of immune tolerance to food substrates and the normal gut microbiome. The main cytokines involved in the formation of immunological tolerance are IL-10 and TGF-beta, which are produced by CD4+ T cells, some populations of macrophages and other cells and have an anti-inflammatory effect, limiting the expansion of effector cells and inducing the proliferation of regulatory T cells[78].

FUNCTIONS OF THE GUT MICROBIOTA AS AN EXTERNAL ORGAN OF THE HUMAN BODY

The microbiota are currently considered an external organ of the human body, which provides important mechanisms of metabolic regulation and protection, alongside the development and functioning of all organ systems[79]. It performs the following functions, the list of which is incomplete.

Digestion of plant polysaccharides. Approximately 17 carbohydrate-active enzymes are formed in the human body, while the microbiota provides around 89[80]. The gut microbiota actively digests dietary fiber, which the human body is unable to digest. These processes take place in the large intestine *via* the most actively involved enzymatic anaerobes, which decompose polysaccharides, particularly representatives of the *Bacteroidaceae* and *Clostridiaceae* families[12,81]. As a result of their digestion, compounds are produced that have a positive effect on the intestinal mucosa. In addition, the mucus layer is an alternative source of glycans for bacteria[12,51,81,82].

Participation in the metabolism of proteins, lipids and fatty acids[83-87]. In particular, gram-negative (*Bacteroides thetaiotamicron*) and gram-positive (*Lactobacillus rhamnosus gg*) bacteria are involved in the regulation of lipid absorption by activating cholecystokinin and secretin receptors expressed by epithelial endocrine cells of the proximal small intestine[88].

Energy supply of IECs[15,81,89] and regulation of their proliferative activity[90,91].

Modulation of goblet cell functions and mucin secretion[16].

The presence of intestinal microorganisms affects the number of Paneth cells and hence the integrity of the epithelial barrier, as Paneth cells regulate ISC homeostasis[35].

Stimulation of local and systemic immunity due to activation of the synthesis of IgA, interferons, and activation of immune cells (macrophages, lymphocytes, and DCs), influence on the development of the intestinal lymphoid apparatus in newborns[92-94].

Synthesis of group B, K vitamins, a number of coenzymes, for example, tocopherols[95-97].

Participation in the regulation of intestinal peristalsis[98-100].

Influence on bone metabolism and pathogenesis of osteoporosis[14,101,102]. The bacterial synthesis of SCFA leads to a decrease in pH in the intestinal lumen and an increase in calcium solubility, an increase in its absorption, and a decrease in bone resorption. *Bifidobacterium* and *Lactobacillus* are mainly involved in these processes. In addition, *Fusobacterium nucleatum* can enhance osteoclast differentiation through increased expression of IL-17A, TNF-alpha, and trimethylamine N-oxide (TMAO), while

Bacteroides, *Lactobacillus*, and *Bifidobacterium* can promote the development of Treg cells and thereby increase osteoblast activity[14,101,103,104].

Influence on the processes associated with the synthesis of neurotransmitters, myelination of neurons in the prefrontal cortex, with the development of the amygdala and hippocampus[105,106]. In dysbacteriosis, the response to antidepressant therapy may be impaired[107]. Germ-free mice show hyperactivity, memory and learning deficits and impaired expression of the serotonin 5-HT1A and NMDA receptors in the hippocampus[108,109].

Inhibition of the growth of pathogenic microorganisms is due to the activation of phagocytosis, the synthesis of antibacterial peptides or the synthesis of bacteriocins that inhibit the growth of competitors [69,73,74,81,100,110].

Influence the effectiveness of several drugs, in particular antibiotics, proton pump inhibitors, metformin, vitamin D and laxatives. It has been shown that the use of these drugs disrupts both the composition of the microbiota and its functional activity[89,111,112].

PRODUCTS OF BACTERIAL METABOLISM AND THEIR ROLE IN HEALTH AND DISEASE

As already noted, health and disease conditions are largely dependent on the functioning microbiome. Products of bacterial metabolism can be crucial for maintaining both the health of an organism and the development of various diseases[113].

SCFAs

The most important products of bacterial fermentation are SCFAs: butyrate, acetate and propionate. The main producers of SCFAs are *Firmicutes* and *Actinobacteria*.

Butyrate: Butyrate is the primary metabolite of *Firmicutes*. It can be synthesized through condensation of 2 molecules of acetyl-CoA, which are reduced to butyryl-CoA and then converted to butyric acid by phosphotransferase and butyrate kinase[114]. It can also be synthesized from butyryl-CoA, lactate and acetate using the acetyl-CoA transferase pathway[115] and from proteins using lysine[116]. It has anti-inflammatory, antitumour, antiproliferative and immunomodulatory properties and is involved in genetic/epigenetic regulation[117,118]. In particular: (1) Regulates antigen-specific adaptive immunity mediated by T- and B-cells: induce T-cells to produce IL-10; regulate the transcription of some cytokine genes, such as IFN-γ and TNF-α, and the activity of the nuclear factor kappa B (NF-κB) signaling pathway; reduce the production of proinflammatory mediators (TNF-α, IL-6, IFN-γ and NO); increase the production of antibodies by B-cells and promote the differentiation of B-cells into plasma B-cells [119-121]; (2) Participates in fat metabolism, reduces insulin resistance, hyperglycaemia, hyperinsulinaemia, and lipid concentrations in the liver and pancreas, thereby reducing the risk of obesity[122]; (3) Affects fatty acid receptors in epithelial, enteroendocrine, neuronal and glial cells, which leads to the production of serotonin by enterochromaffin cells. This may affect the peripheral and central nervous systems of experimental model organisms[50,123]; (4) Helps improve memory[124]; (5) Involved in maintaining the mechanical integrity of the intestinal barrier by inducing the expression of occludin, ZO-1 mRNA and claudin-1 mRNA, thereby reducing intestinal permeability and increasing intestinal villus growth[13,122,125,126]; and (6) Inhibits the rate of cancer cell migration and invasion by increasing the expression of antimetastatic genes (*e.g.*, metalloproteinases) and inhibiting the activation of prometastatic genes (*e.g.*, matrix metalloproteinases)[85,127].

Acetate: Acetate is a fermentation product of various bacteria and is produced from pyruvate using acetyl coenzyme A[128]. It performs the following functions: (1) Participates in the regulation of cholesterol synthesis and activation of local immunity[129]; (2) Helps increasing physical endurance [130]; (3) Influences cognitive functions by activating synaptophysin synthesis[131]; (4) Promotes appetite reduction, fat oxidation, increased levels of proinflammatory cytokines through activated secretion of intestinal hormones such as glucagon-like peptide-1 and peptide YY and increased insulin sensitivity[86]. Nonobese patients show higher production of acetate by gut microbiota than obese patients[132]; (5) Regulates the gut microbiome by increasing the production of IgA and its selective binding to certain microorganisms[86].

Propionate: Propionate is the primary metabolite of *Bacteroidetes* fermentation and is formed from the conversion of succinate to methylmalonyl-CoA by the succinate pathway or from acrylate by the acrylate pathway using lactate as a precursor[133]. Additionally, fucose and rhamnose can be used as substrates for the synthesis of propionic acid *via* the propanediol pathway[134]. Propionate performs the following functions: (1) Participates in maintaining the mechanical integrity of the intestinal barrier by increasing the expression of gut TJ proteins ZO-1, occludin and cadherin[135,136], as well as the synthesis of the antimicrobial protein Regenerating islet-derived protein type 3 (Reg3)[137]; (2) Reduces the risk of developing atherosclerosis and the development of cardiovascular disease by increasing insulin sensitivity and reducing the levels of proinflammatory IL-8[138] and IL-17[139], as well as by reducing the absorption of cholesterol in the intestine by an immune-mediated mechanism through an

increase in the number of regulatory T cells and the level of IL-10, which suppress the expression of C1-like 1 Niemann-Pick (Npc1 L1), the main cholesterol transporter in the intestine[140]; (3) Influences physical endurance[141] and motor functions[136]; (4) Promotes regeneration and functional recovery of sensory axons through an immune-mediated mechanism[142]; and (5) Involved in the regulation of the gut microbiome, possibly through direct suppression of the growth of pathogenic microorganisms[143].

Tryptophan derivatives

A number of other intestinal metabolites can also affect the health of individuals. Among these metabolites, a special place is occupied by tryptophan derivatives: serotonin, tryptamine, kynurenine, and indoles. They perform the following functions.

Act on the central nervous system through the brain-gut axis[144]. The influence is implemented due to the impact on the glutamatergic receptor N-methyl-d-aspartate (NMDA). It has been established that kynurenine, a breakdown product of tryptophan, easily penetrates the blood-brain barrier, where it is metabolized to form neuroactive glutamatergic compounds, kynurenic acid or quinoline acid, acting in the opposite way. Kynurenic acid acts as an NMDA receptor antagonist and has a neuroprotective effect, while quinolinic acid acts as an NMDA receptor agonist and exhibits a neurotoxic effect[145]. In major depressive disorder and bipolar disorder, a decrease in tryptophan and kynurenine was noted. In these mental disorders, there is a shift in tryptophan metabolism from the serotonin pathway to the kynurenine pathway[146]. Tryptophan metabolites are involved in the pathogenesis of various neurodegenerative disorders (Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease) as well as other diseases such as AIDS, cancer, cardiovascular disease, inflammation and irritable bowel syndrome[147,148].

Participate in the regulation of activation, proliferation and migration of immune surveillance cells (T- and B-lymphocytes, macrophages and natural killer cells) and the production of inflammatory signaling molecules, cytokines, nitric oxides and superoxides[149,150].

Influence the motility of the gastrointestinal tract. In particular, as a result of tryptamine action on the serotonin receptor 5-HT4R[151].

Indoles may contribute to the development of cardiovascular, metabolic and psychiatric diseases [152].

Secondary bile acids

It is worth noting that secondary bile acids play a special role in the development of inflammation and colorectal cancer (CRC). It is known that between 5% and 10% of nonreabsorbed bile acids can undergo biotransformation into secondary bile acids as a result of bacterial metabolism involving bacterial bile acid hydrolases (BSHs). Most BSH bacteria are gram-positive enteric bacteria, including *Clostridium*, *Enterococcus*, *Bifidobacterium*, and *Lactobacilli*. The only gram-negative bacteria with BSH activity are members of the genus *Bacteroides*[85,153]. Interestingly, secondary bile acids at high concentrations associated with Western diets can induce oxidative/nitrosative stress, ROS production, DNA damage, apoptosis and mutations[85,154,155] and induce proinflammatory macrophage M1 polarization[154] by binding secondary bile acids with Takeda G protein-coupled receptor 5 (TGR5)[156], thereby initiating inflammation[157]. Several studies have noted that through the activation of the epidermal growth factor receptor (EGFR), secondary bile acids can induce COX-2 expression, stimulate EGFR-MARK signaling[155,158], activate cellular β-catenin signaling and the NF-κB pathway[155,159], and provide transfer of extracellular signal-regulated kinase 1 and 2 *via* activator protein 1 and c-Myc, thereby stimulating the proliferation and invasiveness of colon cancer cells[85,160,161]. At the same time, at low and physiological concentrations, secondary bile acids can have an anti-inflammatory and antitumour effect through a reduction in proinflammatory cytokine levels[162,163]. In particular, an antitumour effect has been noted for lithocholic and ursodeoxycholic acid (UDCA), which are secondary bile acids produced by *Clostridium* species. Lithocholic acid (LCA) at concentrations corresponding to its tissue reference concentrations (< 1 μmol/L) has an antitumour effect on breast cancer cells by inhibiting the epithelial-mesenchymal transition, reducing the production of vascular endothelial growth factor, and activating antitumour immunity[164]. UDCA may prevent the development of CRC by regulating oxidative stress in colon cancer cells[165]. However, the preventive effect of UDCA on CRC is not universally accepted[166].

TMAO

TMAO is a molecule resulting from the oxidation in the liver of a microbial metabolism product, trimethylamine (TMA). TMA is formed in the colon from choline, betaine, and carnitine. The main food precursors of TMA are red meat, fish, poultry, and eggs. TMA from the colon is absorbed into the bloodstream, where it is oxidized by the hepatic enzyme flavin-containing monooxygenase-3 to TMAO, most of which is then excreted unchanged in the urine. Plasma TMAO levels are determined by several factors, including diet, age, gut microbiota, drug intake, and liver flavin monooxygenase activity. The main TMA producers are *Clostridia*, *Shigella*, *Proteus*, *Aerobacter*, and *Eubacterium sp.*[167,168]. Some bacterial enzymes are able to oxidize TMA to TMAO in the colon[169]. At the same time, TMAO can be metabolized into dimethylamine, formaldehyde, ammonia and methane by some methanogenic

bacteria, which leads to its depletion in the colon[170]. It cannot be excluded that the production of formaldehyde under oxidative stress conditions caused by TMAO metabolism may be one of the factors contributing to the induction of CRC. In the experiment, intragastric administration of a suspension of CaCO3 in a mixture with formaldehyde and hydrogen peroxide induced tumors of the stomach and cecum in rats[171].

It has now been established that elevated plasma levels of TMAO correlate with the risk of developing atherosclerosis[172-175], obesity[175,176], cardiovascular diseases[174,177,178], type 2 diabetes[175], chronic kidney disease[179,180] and CRC[181,182]. Elevated TMAO levels have been associated with endothelial dysfunction and inflammatory damage to the vascular endothelium[183, 184], an increase in the level of proinflammatory cytokines, and a decrease in the level of anti-inflammatory cytokines[185,186] with the activation of the MAPK and NF-κB transcriptional pathways[185], oxidative stress[177], cell proliferation and angiogenesis[187]. Xu *et al*[188] provided evidence that these risks may be genetically determined. Moreover, it is worth noting that the effects of TMAO may differ between healthy and diseased conditions. In healthy individuals, TMAO can demonstrate protective, antioxidant or anti-inflammatory effects, while in patients, especially under conditions of oxidative stress, it can have a negative impact on human health[189]. Further research is needed to elucidate the effects of TMAO on human health.

Hydrogen sulfide

Hydrogen sulfide is a metabolite of sulfate-reducing bacteria that metabolize dietary sulfates and other sulfur-containing compounds, including taurine[155]. It is produced by a wide range of *Enterobacteria*, primarily of the genus *γ-Proteobacteria*. The hydrogen sulfide concentration in the large intestine is more than that 5 times higher than in the small intestine[190]. Hydrogen sulfide, similarly to secondary bile acids and TMAO, can multidirectional affect inflammation, oxidative stress, and carcinogenesis[155, 191]. Various authors have demonstrated both its inflammatory[192] and anti-inflammatory effects[193, 194], as well as its carcinogenic[195,196] and anticancer properties[197,198]. For example, some researchers have demonstrated that hydrogen sulfide may be associated with the breakdown of disulfide bonds in the mucus double layer in the colon wall, leading to inflammation and translocation of bacteria and toxins[199]. At the same time, other researchers suggest that hydrogen sulfide can protect the mucus layer and repair this already destroyed, thereby preventing inflammation[190]. It is believed that the physiological and pathological effects of hydrogen sulfide are associated with its concentration[200]. Given that intracellular hydrogen sulfide has a significant impact on many cellular functions, such as TJs, autophagy, apoptosis, vesicle trafficking, cell signaling, epigenetics and inflammasomes[201], and can be used as a therapeutic agent, its further study may open new opportunities in understanding the mechanisms of the development of pathological conditions and their treatment.

Polyamines

Polyamines are versatile polyfunctional molecules involved in cell proliferation and differentiation, apoptosis, angiogenesis, immune response, signaling, and gene expression[202,203]. They can be supplied with food and can form as a result of endogenous synthesis, as well as a result of bacterial metabolism, such as putrescine, spermidine and cadaverine[204,205]. In particular, cadaverine is formed from lysine by decarboxylation with the participation of lysine decarboxylase (LDC). Putrescine is formed by decarboxylation of ornithine catalyzed by ornithine decarboxylase. Spermidine synthase is involved in the formation of spermidine from putrescine. These enzymes are produced by most gram-negative bacteria[205,206]. Polyamines synthesized by the intestinal microbiota are then transported into the bloodstream through the colonic mucosa[207].

It has been established that polyamines produced by intestinal bacteria suppress chronic inflammation and strengthen the intestinal barrier in the colon, contribute to a significant improvement in the host's cognitive functions and increase life expectancy in experimental animals and have a cardioprotective effect[203,208,209]. In a number of studies, the antitumour effect of cadaverine was noted. In an experiment, cadaverine caused a decrease in the proliferative activity and invasiveness of breast cancer cells and contributed to the induction of mesenchymal-epithelial transition, a decrease in the stemness of cancer cells and their ability to metastasize[210]. In breast cancer, a decrease in the intestinal biosynthesis of cadaverine was noted, especially in patients with carcinoma in situ and stage I of the disease. With a high expression of bacterial LDC in the gut contents, a significantly longer survival was noted than with a low expression[210].

At the same time, a number of studies have noted that high levels of polyamines may be associated with tumor transformation and cancer progression[211]. Thus, in CRC, an increase in the levels of bacterial cadaverine and putrescine in the feces was noted[212,213]. Huang *et al*[214] reported that increased spermine intake is associated with an increased risk of CRC, while a higher intake of total polyamine, putrescine and spermidine is significantly associated with a reduced risk of CRC. It is believed that the procarcinogenic effect of polyamines is associated with the activation of the PTEN-PI3K-mTOR (TORC1), WNT, and RAS pathways[211].

Microbiome and biotransformation of xenobiotics

The gut microbiome can influence the biotransformation of a number of xenobiotics with known carcinogenic properties, such as heterocyclic amines (HCAs). HCAs are formed during thermal processing (frying, baking, grilling, *etc.*) of various food products, including oils, grains and vegetables, but especially processed meat[215]. HCAs have pronounced genotoxic and mutagenic properties, contributing to the development of malignant neoplasms of the intestine, liver, lungs, breast and other tumors. The carcinogenicity of HCAs is associated with mutations in proto-oncogenes and tumor suppressor genes, including K-ras, Haras, Apc, β-catenin and TP53[216]. The intestinal microbiota can metabolize HCAs into molecules with increased mutagenic activity[217]. At the same time, the intestinal microbiota can bind or metabolize food-derived HCAs, facilitating their excretion with feces or conversion into less toxic compounds[215,218]. These processes involve bacterial beta-glucuronidase (B-GUS) and glycerol/diol dehydratase (GDH) produced by some lactic acid bacteria and probiotics. A decrease in the number of taxa with B-GUS and GDH activity was noted in patients with CRC[219].

DYSBIOSIS AND HUMAN DISEASES

According to the results of numerous studies, in many diseases, including inflammatory bowel diseases [113,220-224], chronic liver diseases[225-227], obesity[228,229], diabetes mellitus[230,231], osteoporosis [41,232], cardiovascular[233,234] and oncological diseases[212,213,235], a decrease in the microbiota diversity and an overgrowth of pathogenic and conditionally pathogenic flora are observed[50,236,237]. Interestingly, in CRC, *Fusobacterium nucleatum* was found not only in the primary tumor but also in metastatic lesions in the liver[238] and lung[239]. In addition, it has been noted that dysbiosis to a certain extent can influence the development of depression, bipolar depression and schizophrenia[240], as well as autism[241] and Parkinson's disease[242,243]. Figure 1 demonstrates the association of gut dysbiosis with various human diseases.

It should be emphasized that the diseases associated with dysbacteriosis are multifactorial in nature [50,236,237,244] and are associated with bacterial invasion through the physical and chemical barriers of the gastrointestinal tract[50]. It is hypothesized that diet and other environmental influences may change the microbiome and thus provoke an unstable basis of genetic predisposition, which may lead to disease development, at least in some patients. Dysbiosis may be related to heredity, use of antibiotics, proton pump inhibitors, certain types of chemotherapy, advanced age, diet, and other factors[245,246]. At the same time, it should be noted that numerous studies have not revealed typical changes in the microbiome for a particular pathology[247]. A detailed analysis of host metabolism (metabolic index) and habitual diet (including the consumption of plant and animal foods, and fermented milk probiotics, such as yogurt) allowed Asnicar *et al*[248] to establish consistent gut microbiome signatures, segregating favourable and unfavourable taxa with multiple measures of both dietary intake and cardiometabolic health. However, we believe the issue cannot be considered definitively resolved, since it is not completely clear what is primary: A violation of the microbiome that then contributes to the development of diseases or disorder can cause changes in the microbiome. These questions require further research.

MODERN APPROACHES TO THE REGULATION OF THE GUT MICROBIOME

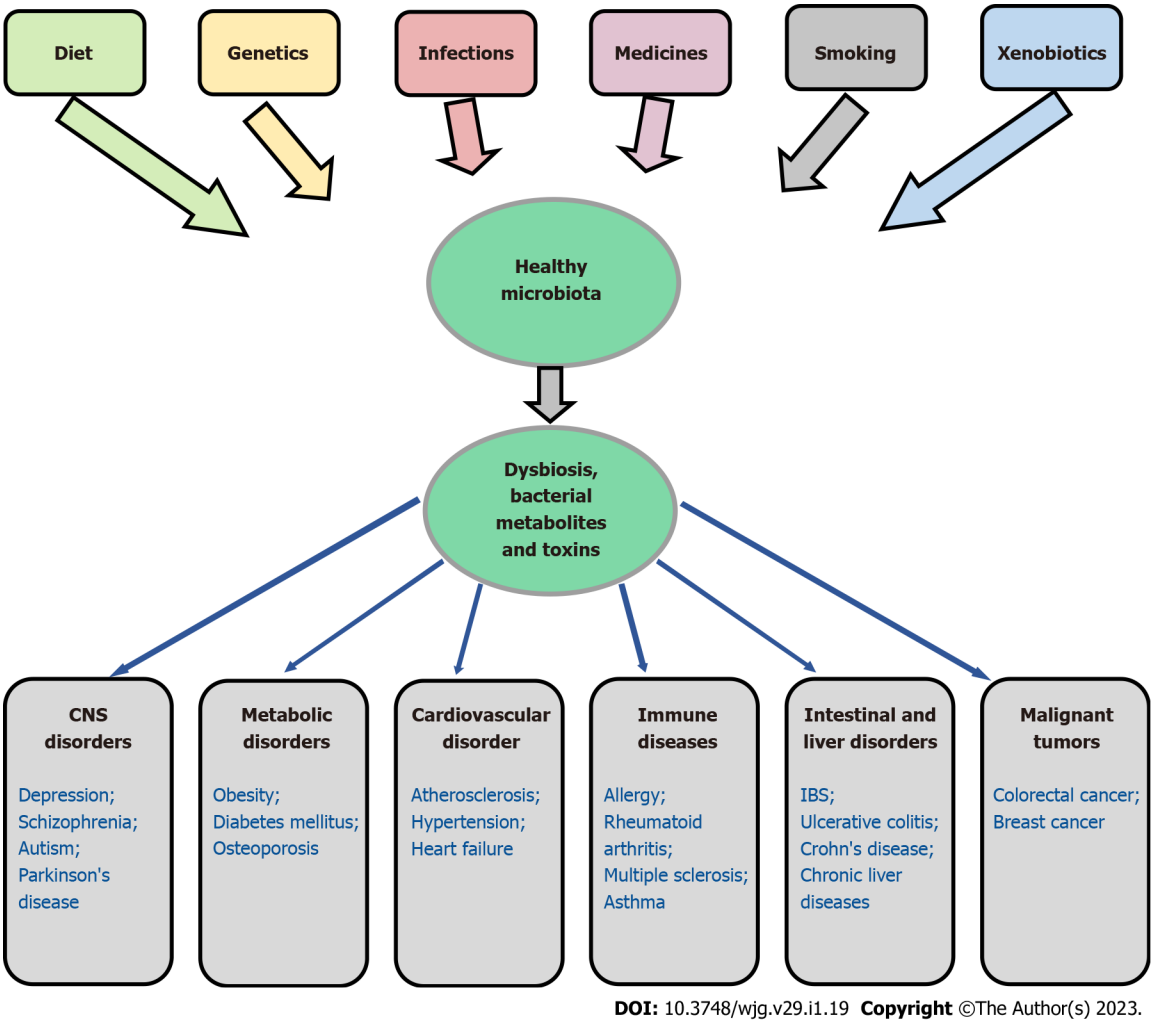
Considering that intestinal dysbiosis plays an important role in the development of a number of diseases, normalizing the microbiome seems to be a promising direction for their treatment. There are several approaches to solve this problem.

"Mediterranean diet"

It is well known that a Mediterranean diet is rich in vegetables, fruits, whole grains and fish and thus creates favourable conditions for beneficial bacteria such as *Firmicutes*, which are involved in the production of butyrate necessary to maintain a healthy barrier between the colon and blood flow, preventing inflammation in the gut[64]. Unlike the Mediterranean diet, a “Western” high-fat, low-fiber diet contributes to colon inflammation and cancer[249,250].

Prebiotics

Prebiotics are substrates that are selectively utilized by host microorganisms to provide health benefits [251]. The use of prebiotics, such as dietary fiber, reduces obesity and has anti-inflammatory and anticancer effects[85,250,252,253]. Dietary fiber intake is associated with a lower incidence of colon cancer, since fiber reduces the concentration of intestinal carcinogens due to increased stool mass, intestinal motility and production of butyrate, which maintain colonocyte health, increase apoptosis and inhibit cancer cell proliferation. A long-term fiber-rich diet increases the density of *Firmicutes*, which have the ability to mediate an immunomodulatory and anti-inflammatory immune response[64].



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Figure 1 Association of gut dysbiosis with various human diseases. CNS: Central nervous system; IBS: Irritable bowel syndrome.

Moreover, dietary fiber physically interferes with fatty acid reabsorption and cholesterol absorption, thereby reducing the risk of obesity, diabetes, and atherosclerosis[254,255].

Transplantation of fecal microbiota

The mechanism of transplantation of fecal microbiota (FMT) is to restore the fermentation activity, pH and redox potential of the microbiome habitat in the respective niches and restore normal gas production and synthesis of SCFA[245,256]. FMT has been used in the treatment of nosocomial diarrhea caused by *Clostridioides difficile*[245] and other intestinal diseases[257-259]. Scibelli *et al*[260] demonstrated the effectiveness of using FMT in the treatment of rectal fistula in a patient with a colostomy who received intensive antibiotic therapy for a long time due to trauma. Impressive results have also been obtained in the treatment of Crohn's disease by FMT: nearly 60% of patients achieved a clinical response to treatment, and more than 20% of patients experienced sustained clinical remission, including 2 of 6 patients with perianal fistula[261]. The use of FMT has been shown to be beneficial in hepatic encephalitis, metabolic diseases, neuropsychiatric disorders, autoimmune diseases, allergic disorders, tumors, Parkinson's disease, multiple sclerosis, myoclonus dystonia, chronic fatigue syndrome, and idiopathic thrombocytopenic purpura[257,262-264].

The disadvantages of FMT are frequent side effects such as constipation, diarrhea, bloating and possible transmission of potential pathogens[245,265]. Given the enormous promise of microbiota transplantation, the search for new methods and ways to use it continues[266]. Attempts have been made to replenish only bacteria that had certain characteristics and whose number was reduced[245,267,268], but reproducibility and standardization of preparations used for transplantation have been proven to be a problem[267]. Zhang *et al*[269] first revealed that washed microbiota transplantation is safer, more precise and more quality-controllable than crude FMT by manual. Currently, there are a series of clinical trials conducted for SER-109, which is a consortium from several species of *Firmicutes* isolated from the stool of healthy human donors and encapsulated. The use of the medication reduced the risk of recurrence of nosocomial diarrhea caused by *Clostridioides difficile* from 41.3% in the placebo group to 11.1% in the treatment group[270].

Probiotics

Probiotics are live strains of carefully selected microorganisms that, when administered in adequate amounts, confer a health benefit on the host[271]. Probiotics can be easily and economically prepared and given to patients daily in the form of yogurt, drinks, cheese or capsules[272]. They contribute to the maintenance of intestinal barrier function, have immunomodulatory, metabolic and antiproliferative effects[273,274], and regulate DC maturation by producing tolerogenic DCs, which can reduce inflammation[275] and synthesize antimicrobial substances[276]. The ability of *Bifidobacterium dentium* and its secreted factors to suppress endoplasmic reticulum stress genes and promote MUC2 secretion, as well as to secrete the antioxidant γ-glutamylcysteine, which reduces the formation of ROS and suppresses NF-kB activation and IL-8 secretion, has been established[277].

Currently, *Lactobacillus*, *Bifidobacterium* and *Saccharomyces* are used to treat hospital-acquired diarrhea caused by *Clostridioides difficile*[278]. Oral use of *Lactobacillus reuteri 6475* reduced the loss of total bone mineral density (BMD) in women aged 75 years to 80 years with low BMD[279]. Dietary supplementation with soluble corn fiber at doses of 10-20 g/d has also been associated with an increase in calcium absorption and a larger number of *Clostridium* and unclassified *Clostridiaceae* in feces[280]. These data suggest that both probiotics (live bacteria) and dietary supplements needed to feed the bacteria can be used as therapeutic agents to combat osteoporosis. It is possible that the use of their combination will be even more effective.

For therapeutic purposes, commensals (bacterial strains that are resistant to certain types of pathogenic bacteria), bacteriophages and fungi can be used. For example, CBM588 is a probiotic composed of the commensal *Clostridium butyricum*, which produces large amounts of butyrate and activates neutrophils and Th1 and Th17 cells[281]. Bacteriophages are viruses that can infect and multiply in pathogenic bacteria and eventually lyse them[245,282]. Yeasts such as *Saccharomyces boulardii* and *Candida albicans*, as well as fungal wall components such as β-glucans, can inhibit the growth of some enteric pathogens. *Saccharomyces boulardii* produces proteases or phosphatases that inactivate disease-causing toxins produced by gut bacteria and modulate multiple signaling pathways to suppress toxin-induced inflammation[245,283,284]. Because of the risk of fungemia, they should be used cautiously in debilitated patients[285].

CONCLUSION

The study of the influence of the gut microbiome on health and disease is one of the most relevant and interesting areas in modern science. The number of microorganisms inhabiting the human body is enormous, and their composition can vary significantly between individuals[248,286]. Dysbiosis has been found to play an important role in the development of a number of diseases, however, numerous studies have not revealed typical changes in the microbiome characteristic for a particular pathology [247]. It is likely that the impact of the microbiome and its metabolites on human health cannot be considered only in terms of health benefits or harms. Metabolites that perform important functions in the human body under certain conditions can have a negative impact on human health, while metabolites that are considered potentially dangerous can be beneficial[287].

As already noted, changes in the quantitative and qualitative microbiota composition can lead to an increase in the production of potentially toxic metabolites, such as secondary bile acids, TMAO and hydrogen sulfide, and an increase in the risk of developing intestinal, cardiovascular, neurological, oncological and other diseases. These changes may be related to diet, lifestyle, age, medications, and other factors. Thus, taking antibacterial drugs can increase sensitivity to viral infections[288], increase the risk of developing malignant neoplasms[289], and contribute to the development of resistance to chemotherapy drugs and immune checkpoint inhibitors in cancer patients[290]. Interestingly, some authors attribute an increase in the risk of malignant neoplasms with the use of antibiotics to a decrease in the synthesis of intestinal metabolites with antitumour activity, for example, LCA and cadaverine [289]. However, in some malignant neoplasms, the antitumour effect of antibacterial drugs was demonstrated. For example, in CRC, antibiotic therapy had a cytostatic effect due to the destruction of bacterial biofilms, the formation of which was associated with polyamines[291]. In an experiment, adding broad-spectrum antibiotics to drinking water for 3-4 wk reduced age-related oxidative stress and arterial dysfunction in mice[292]. It is believed that microbiota modulation using antibiotics, probiotics, fecal microbiota transplantation or nanotechnology can be effective in a variety of diseases, including enhancing the antitumour effect of chemotherapy drugs or immune checkpoint inhibitors [290].

It should be noted that despite the huge amount of research on the role of the microbiome in health and disease development, there are a number of issues that deserve further study. In particular, it is known that the microbiota affects the mood and behavior of a person, his physical activity, resistance to stress and diseases. At the same time, whether mental and physical health influence the human microbiome is not well understood. Many questions remain regarding the role of the gut microbiome in drug metabolism, the development of drug resistance and chemoresistance, and the role of the microbiome in cancer progression. Answers to these and other questions are still waiting for their

researchers.

FOOTNOTES

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REVIEW

Current status and future perspectives of radiomics in hepatocellular carcinoma

Joao Miranda, Natally Horvat, Gilton Marques Fonseca, Jose de Arimateia Batista Araujo-Filho, Maria Clara Fernandes, Charlotte Charbel, Jayasree Chakraborty, Fabricio Ferreira Coelho, Cesar Higa Nomura, Paulo Herman

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Joao Miranda, Department of Radiology, University of Sao Paulo, Sao Paulo 05403-010, Brazil

Natally Horvat, Maria Clara Fernandes, Charlotte Charbel, Department of Radiology, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, United States

Gilton Marques Fonseca, Fabricio Ferreira Coelho, Paulo Herman, Department of Gastroenterology, University of Sao Paulo, Sao Paulo 05403-000, Brazil

Jose de Arimateia Batista Araujo-Filho, Department of Radiology, Hospital Sirio-Libanes, Sao Paulo 01308-050, Brazil

Jayasree Chakraborty, Department of Surgery, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, United States

Cesar Higa Nomura, Department of Radiology, University of Sao Paulo, Sao Paulo 05403-000, Brazil

Corresponding author: Paulo Herman, MD, PhD, Professor, Department of Gastroenterology, University of Sao Paulo, 255 Av. Dr. Eneas de Carvalho Aguiar, Sao Paulo 05403-000, Brazil. pherman@uol.com.br

Abstract

Given the frequent co-existence of an aggressive tumor and underlying chronic liver disease, the management of hepatocellular carcinoma (HCC) patients requires experienced multidisciplinary team discussion. Moreover, imaging plays a key role in the diagnosis, staging, restaging, and surveillance of HCC. Currently, imaging assessment of HCC entails the assessment of qualitative characteristics which are prone to inter-reader variability. Radiomics is an emerging field that extracts high-dimensional mineable quantitative features that cannot be assessed visually with the naked eye from medical imaging. The main potential applications of radiomic models in HCC are to predict histology, response to treatment, genetic signature, recurrence, and survival. Despite the encouraging results to date, there are challenges and limitations that need to be overcome before radiomics implementation in clinical practice. The purpose of this article is to review the main concepts and challenges pertaining to radiomics, and to review recent studies and potential applications of radiomics in HCC.

Key Words: Radiomics; Hepatocellular carcinoma; Texture analysis; Radiology

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Core Tip: Radiomics is an emerging field that extracts high-dimensional mineable quantitative features that cannot be assessed visually with the naked eye from medical imaging. The main potential applications of radiomic models in hepatocellular carcinoma (HCC) are to predict histology, predict response to treatment, predict genetic signature, predict recurrence, and predict survival. The purpose of this article is to review the main concepts and challenges pertaining to radiomics, and to review recent studies and potential applications of radiomics in HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related deaths worldwide [1]. Liver cancer is especially common in Asia, where 72.5% of all new liver cancer cases worldwide are diagnosed[2]. HCC accounts for over 90% of all primary liver cancer cases[3]. The main risk factors for HCC in the West is viral hepatitis (hepatitis C virus in the West and hepatitis B virus in Asia and in developing countries) and alcohol intake. In addition, non-alcoholic steatohepatitis is becoming a common risk factor, particularly in the West[3,4]. HCC patient prognosis depends on the stage of HCC at the time of diagnosis[5]; and advanced-staged patients at the time of diagnosis have a poor prognosis [5-7].

The treatment of HCC is based on tumor burden, clinical performance of the patient, and liver function[8]. Given the frequent co-existence of an aggressive tumor and underlying chronic liver disease, the management of HCC requires experienced multidisciplinary team discussion[9]. Moreover, radiology plays a key role in the screening, diagnosis, staging, restaging, and surveillance of HCC. Currently, imaging assessment is based on qualitative characteristics, such as size and enhancement pattern, which are prone to inter-reader variability. Reliable tools that can potentially address this variability as well as deal with the vast amount of imaging data are warranted[10]. Over the last decade, radiomics has become a popular quantitative tool that can potentially address these challenges and provide information not previously available for precision decision-making[11].

Radiomics is an emerging field that extracts high-dimensional mineable quantitative features that cannot be assessed visually with the naked eye from medical imaging[12]. The main potential applications of radiomic models in HCC are to predict histology, response to treatment, genetic signature, recurrence, and survival[13]. Despite the encouraging results to date, there are several challenges and limitations that need to be overcome before the implementation of radiomics in clinical practice. The purpose of this study is to review the main concepts, challenges pertaining to radiomics and recent studies and potential applications of radiomics in HCC.

RADIOMICS

Main concepts

In the new era of precision medicine, artificial intelligence (AI) and in its various branches, such as machine learning (ML) and deep learning (DL), have provided new imaging biomarkers that can potentially provide new data that are useful for clinical decision-making. ML is related to a set of computational systems that improve with experience. DL is a subset of ML based on series of layers (trainable nonlinear operations), each of which transforms input data into a representation that facilitates pattern recognition[14].

Radiomics has recently emerged as a translational research field that proposes to discover new associations between clinical data and quantitative data extracted from medical images using conventional biostatistics or AI methods[12] and become popular, particularly in oncologic imaging. Radiomics involves mineable high-dimensional data extraction, characterizing intensity, shape, size, and/or texture from images to create big-data datasets that are then used to identify distinct sub-visual imaging

patterns[15]. Radiomics models usually use magnetic resonance imaging (MRI), computed tomography (CT) and positron emission tomography (PET) images data. Fundamentally, radiomics is motivated by the observation that these imaging characteristics reflect phenotype and genotype of underlying tissue and thus can help in clinical decision making[16].

Radiomic can be subdivided into texture, size and shape, and transformed based features. The most common radiomic features is texture. It can be subdivided into first-order, second-order and higher-order statistical features. First-order features reflect the distribution of values of individual voxels without concern for spatial relationships; they are generally histogram-based, such as mean (average intensity), entropy (quantify randomness of intensity), kurtosis (flatness) and skewness (asymmetry). Second-order features reflect the statistical interrelationships between voxels with similar (or dissimilar) contrast values[12] and some of the commonly used 2nd order features are: Grey level co-occurrence matrix, grey level run length matrix, and grey level size zone matrix features. Taking into account the repetitive patterns in radiological images, higher-order statistical methods use sophisticated filter grids on the images - such as Minkowski functionals (to evaluate voxels whose intensity is above a determined threshold), Wavelet and Laplacian transforms (to identify coarse texture patterns) and fractal analysis (to assess the irregularity of a surface)[12]. In practice, standard libraries with predefined feature configurations and validated reference values (such as PyRadiomics) are frequently used to increase the reproducibility of radiomic models.

Workflow

Radiomic analysis is a multistep process involving the processing of medical images to generate different features from segmented images. The typical radiomics workflow can be summarized in the following steps (Figure 1):

Image acquisition and preprocessing: Standardized imaging protocols should be used to avoid reproducibility issues related to noise and confounding. However, standardized imaging protocols also decrease the generalizability of the results. Once a patient dataset has been identified, images should be anonymized as well as exported as Digital Imaging and Communication in Medicine files[17]. De-noising and motion correction steps may be needed.

Segmentation: Segmentation involves the delineation of region of interests (ROIs) on the tumor or peritumoral zones. ROIs can be delineated manually, semiautomatically, or automatically (using ML tools) in either two-dimensional (2D) or three-dimensional (3D) views (Figure 2). Whenever possible, segmentations should be checked by a radiologist to ensure accuracy.

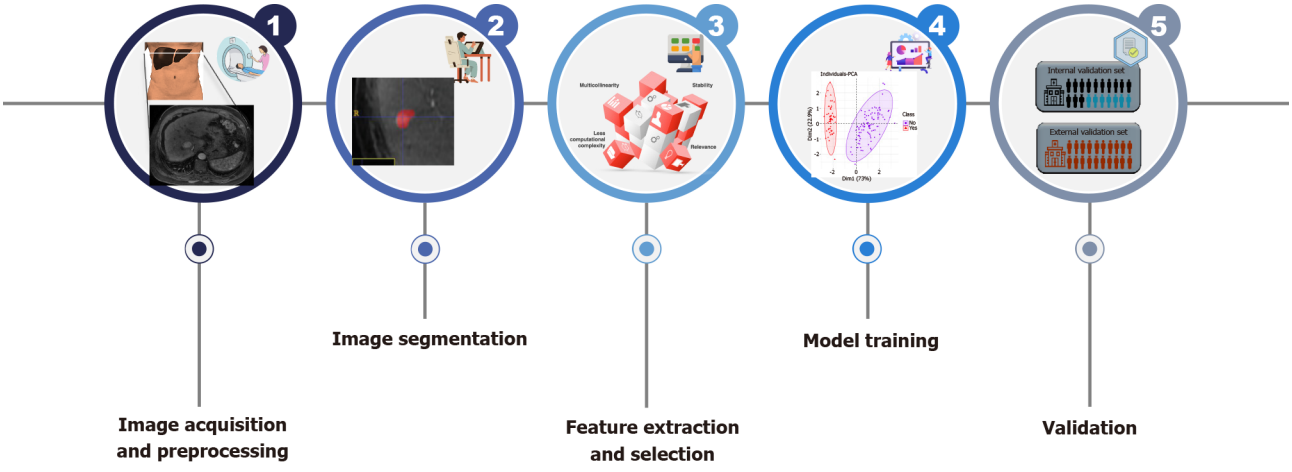
Feature extraction, feature selection and model building: A wide range of statistical models are commonly used to choose a subset of optimal features that correlate with the predenid outcome[15]. Many of the extracted features are in fact redundant and supervised or unsupervised approaches can be applied to achieve dimension reduction. ML and DL techniques are emerging as useful tools to achieve more accurate feature selection[18,19]. The features should be selected only based on the training data to avoid bias.

Of note, the number of extracted features is commonly larger than the study sample, which can contribute to overfitting of the model and to overoptimistic results. Some strategies can be done, for example, select the features in such a way to maintain the ratio or regularization methods are used to minimize the complexity of the respective models[20]. Once the optimal features are identified, a statistical model can be proposed to predict a specific clinical question using different classifiers such as generalized linear models, random forests, support vector machines, or neural networks[20,21].

Validation: Validation is essential to estimate model performance and can be done using subsets of the original training dataset (*i.e.*, cross-validation) or using a separate hold-out dataset containing either internal or external data[17].

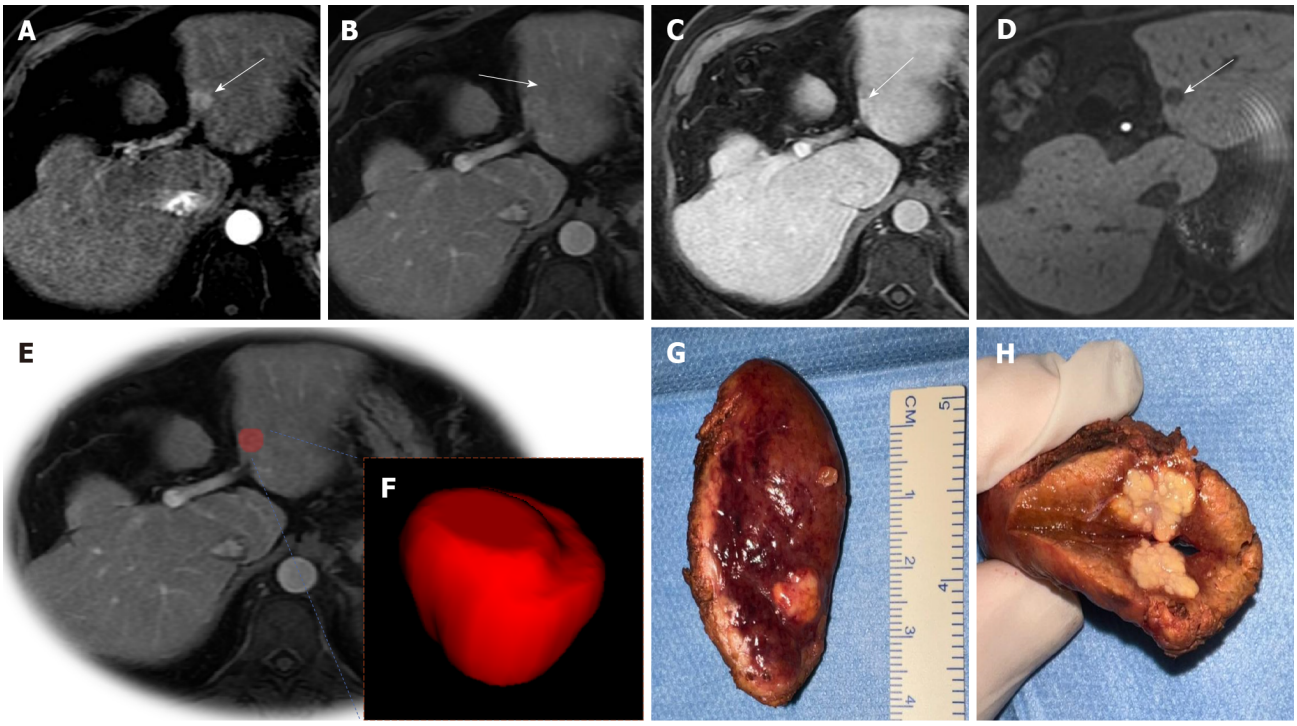
Main challenges

To date, radiomic models reproducibility is often poor, due to insufficient reporting or limited open-source code and data, which undermines external validation and increases the subsequent risk of false-positive results[22]. Further, researchers often face great difficulty in acquiring unbiased and homogeneous datasets across multiple institutions, thus hampering multi-institutional collaborations involving large multi-institutional datasets for the training and validation of radiomic models[14]. For successful multi-institutional cooperation for building large multi-institutional datasets for radiomic models training and validation, radiomics workflow standardization, clear reporting of study methodology, and data sharing across different institutions are needed[17]. Additionally, an effective means to interpret the vast and varied data derived from radiomics analysis is another key obstacle to the clinical implementation of radiomic models. Therefore, a balanced interpretation of results and an increased focus on interpretable models are essential to their successful integration into clinical practice [23]. Finally, manual segmentation is a time-consuming process and one of the most common limitations that should be managed with automatic or semiautomatic strategies before widespread use of radiomics tools.



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Figure 1 Illustration summarizing radiomics workflow.



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Figure 2 Illustration of hepatocellular carcinoma segmentation. 72-year-old man with cirrhosis had a new liver lesion on computed tomography, indeterminate. Gadoxetic acid-enhanced T1-weighted images show a 1.3 cm (arrows) lesion. A: With arterial phase hyperenhancement; B: Questionable washout appearance on portal venous; C: Delayed phases; D: Hypointensity on during hepatobiliary phase (20 min); E and F: A tumor bed segmentation was exemplified, the portal venous phase (E) was used to manually segment the volume of interest (F); G and H: Note the gross findings after surgery. Histology confirmed hepatocellular carcinoma.

APPLICATIONS OF RADIOMICS IN HCC

Prediction of HCC histology

Table 1 summarizes the studies in the literature to date that have evaluated the use of radiomics to preoperatively predict HCC histology.

Distinguishing between HCC and other malignant or benign lesions: The distinction between HCC and other primary hepatobiliary malignancies can be challenging on imaging, because of the overlap of some features, especially for combined tumors[24]. In light of this, many studies have investigated radiomics performance in differentiating HCC from other malignant and benign hepatic lesions. For instance, Liu *et al*[24] studied the use of MRI- and CT-based radiomics to differentiate between HCC,

Table 1 Summary of the studies that evaluated radiomics to preoperatively predict hepatocellular cholangiocarcinoma histology

Ref.	Country	*n*	Imaging modality	Endpoint	Segmentation	ROI/VOI	No. of readers	Main results	Validation
Wang *et al* [92], 2022	China	196	MRI	cHCC-CC *vs* HCC	Manual, intratumoral	ROI	1	AUC (delayed phase MRI): 0.91	None
Liu *et al* [24], 2021	Canada	85	MRI and CT	cHCC-CC *vs* HCC *vs* CC	Manual, intratumoral	ROI	2	AUC (MRI): 0.77-0.81. AUC (CT): 0.71-0.81	Cross-validation
Lewis *et al* [25], 2019	United States	63	MRI	cHCC-CC *vs* HCC *vs* CC	Manual, intratumoral	VOI	2	AUC (LI-RADS and male gender): 0.90	None
Nie *et al* [27], 2020	China	156	CT	HCC *vs* FNH	Manual, intratumoral	ROI	2	AUC (radiomics): 0.96 training, 0.87 validation. AUC (radiomics + clinical factors): 0.98 training, 0.92 validation	None
Wu *et al* [28], 2019	China	369	MRI	HCC *vs* hemangioma	Manual, intratumoral	ROI	2	AUC: 0.86 training, 0.89 testing	None
Mokrane *et al*[29], 2020	United States	178	CT	HCC diagnosis	Manual, intratumoral	VOI	2	AUC: 0.70 training, 0.66 validation	External
Brancato *et al*[34], 2022	Italy	38	MRI	Tumor grade	Manual, intratumoral	VOI	1	AUC: 0.89	None
Gao *et al* [93], 2018	China	Training: 125. Validation: 45	MRI	Tumor grade	Manual, intratumoral	N/A	N/A	AUC: 0.83 training, 0.74 validation	None
Wu *et al* [30], 2019	China	Training: 125. Validation: 45	MRI	Tumor grade	Manual, intratumoral	ROI	1	AUC: 0.83 training, 0.74 validation	Internal
Zhou *et al* [94], 2017	China	46	MRI	Tumor grade	Manual, intratumoral	ROI	1	AUC: 0.83-0.92	None
Mao *et al* [31], 2022	China	Training: 85. Validation: 37	MRI	Tumor grade	Manual, intratumoral	ROI	2	AUC: 0.97 training, 0.94 validation	Internal
Chen *et al* [33], 2021	China	Training: 112. Validation: 49	CT	Tumor grade	Manual, intratumoral	VOI	2	AUC: 0.90 training, 0.94 validation	Internal
Yang *et al* [95], 2019	China	Training: 146. Validation: 62	Gadoxetic acid-enhanced MRI	MVI	Manual, intratumoral	ROI	2 (consensus)	AUC: 0.94 training, 0.86 validation	Internal
Xu *et al* [39], 2019	China	495	CT	MVI	Semi-automatic, intratumoral and peritumoral	VOI	3	AUC: 0.91 training, 0.89 validation	Internal
Feng *et al* [40], 2019	China	160	Gadoxetic acid-enhanced MRI	MVI	Manual, intratumoral and peritumoral	VOI	3	AUC: 0.85 training, 0.83 validation	Internal
Zheng *et al*[41], 2017	United States	120	CT	MVI	Semi-automatic	ROI	1	AUC: 0.80	None
Bakr *et al* [96], 2017	United States	28	CT	MVI	Manual, intratumoral	ROI	4	AUC: 0.76	None
Ma *et al* [97], 2019	China	157	CT	MVI	Manual, intratumoral	VOI	1	AUC (portal venous phase CT): 0.79	Cross-validation

AUC: Area under the curve; cHCC-CC: Combined hepatocellular cholangiocarcinoma; CT: Computed tomography; FNH: Focal nodular hyperplasia; HCC: Hepatocellular carcinoma; MRI: Magnetic resonance imaging; MVI: Microvascular invasion; ROI: Region of interest; VOI: Volume of interest.

cholangiocarcinoma, and combined HCC-cholangiocarcinoma. Using MRI, radiomic features derived from contrast-enhanced phases demonstrated excellent performance to differentiate HCC from non-HCC [area under the curve (AUC) ≥ 0.79], with the highest AUC obtained from the arterial phase (AUC of 0.81); meanwhile, using CT, radiomic features derived from the pre-contrast and portal venous phase yielded AUC values of 0.81 and 0.71, respectively. In another study, Lewis *et al*[25] found that the combination of the apparent diffusion coefficient 5th percentile radiomic feature with Liver Imaging Reporting and Data System classification and male gender achieved an accuracy of 80%-81.5% in distinguishing HCC from intrahepatic cholangiocarcinoma (ICC) and combined HCC-ICC, and outperformed either measure alone. Other studies showed that radiomics is helpful to distinguish between HCC and benign tumors in non-cirrhotic livers, *e.g.*, from hepatocellular adenoma (AUC of 0.96 in the training set and 0.94 in the test set)[26], from focal nodular hyperplasia (AUC of 0.979 in the training set and 0.917 in the test set)[27], and from hemangioma (AUC: 0.86 in the training set and 0.89 in the test set)[28]. Mokrane *et al*[29] validated a radiomics signature to diagnose HCC in patients with cirrhosis and increased radiologists' confidence.

Prediction of histologic grade: Histologic grade is an important prognostic factor in patients with HCC and is only available preoperatively in patients who undergo biopsy. Therefore, studies have aimed to identify non-invasive imaging features such as radiomic features that could potentially predict the tumor grade. Wu *et al*[30] found that MRI-based radiomics can successfully categorize low-grade and high-grade HCC, with the radiomic model outperforming the clinical model (AUC 0.742 for the combined T1-weighted and T2-weighted MRI-based radiomic model *vs* AUC 0.6 for the clinical one) and the combined radiomic and clinical model (AUC 0.8) outperforming both models alone. Mao *et al* [31] also investigated MRI-based radiomic features, with Gd-EOB-DTPA contrast administered for the MRI exams, finding that the artificial neural network combining radiomic features from the contrast-enhanced arterial phase and hepatobiliary phase yielded the highest AUC of 0.944. Moreover, they found that the artificial neural network models were superior to the logistic regression models. In other studies, CT-based radiomics has been found to have high performance in distinguishing between low- and high-grade tumors[32-34]; for instance, Chen *et al*[33] found an AUC of 0.937 for a ML-based radiomics model based on the CT portal phase.

Prediction of microvascular invasion: Microvascular invasion (MVI) is found in about 15%-57% of patients with HCC who undergo surgery[35,36] and is associated with higher rates of recurrence and shorter survival after surgery[37]. Although imaging can be used to diagnose macrovascular invasion (or tumor in vein), preoperative imaging identification of MVI is difficult. Studies have evaluated the performance of radiomics as a tool to predict MVI, with most predictive models combining radiomics and clinical biomarkers[38]. For instance, Xu *et al*[39] proposed a model combining CT-based radiomic features with radiologic and clinical parameters; the model was not only an independent predictor of histologic MVI (AUC of 0.909 in the training/validation set and 0.889 in the test set) but was also an independent predictor of worse prognosis (disease-specific recurrence and disease-specific mortality). Of note, the radiomics-only model did not add significant value to radiologist scores alone. Since MVI occurs primarily at the tumor periphery (approximately 85% of MVI is located within one centimeter from the tumor margin), studies have investigated radiomic features derived from the peritumoral tissue. For instance, Feng *et al*[40] demonstrated that a model combining intratumoral and peritumoral radiomic features was superior in predicting MVI using Gd-EOB-DTPA-enhanced MRI compared to the model containing only intratumoral radiomics features. Additionally, Zheng *et al*[41] demonstrated that peritumoral textural features had an AUC of 0.80 and a multivariate model combining alfa-fetoprotein, tumor size, hepatitis status and quantitative features achieved an AUC of 0.88.

Prediction of HCC genetic expression

Compared to the prediction of histology, fewer researches in the literature have evaluated the use of radiomics to predict genetic expression in patients with HCC (Table 2). Overall, studies on the use of radiomics to predict genetic expression have focused on using radiomics to predict Ki67 expression as well as cytokeratin 19 (CK19), P53, and phosphatidylinositol-3 kinase (PI3K) status. Of note, in 2007, Segal *et al*[42] investigated for the first time the correlation between HCC genetic expression and CT imaging traits, finding 32 CT imaging traits that were correlated with the expression levels of 116 genetic markers.

Ki67 expression: High Ki-67 expression in HCC patients is associated with fast progression and poor prognosis[43]. To determine if radiomics can be useful to predict Ki67 expression, Wu *et al*[44] developed and validated a radiomic nomogram based on the combination of CT-based radiomic features and clinical factors. Using Gd-EOB-DTPA-enhanced MRI, Li *et al*[45] found that texture analysis of the hepatobiliary phase, arterial phase, and portal vein phase were helpful for predicting Ki67 expression. In their study, a single slice with the largest proportion of the lesion was delineated, and the predictive performance of models were compared by misclassification rate. In another study by Fan *et al* [46] using Gd-EOB-DTPA-enhanced MRI, the authors delineated the whole lesion, and the predictive performance of different models were compared using the receiver operating curve, calibration curve,

Table 2 Summary of the studies that evaluated radiomics models to predict genetic profile in patients with hepatocellular cholangiocarcinoma

Ref.	Country	*n*	Imaging modality	Endpoint	Segmentation	ROI/VOI	No. of readers	Main results	Validation
Xia *et al* [98], 2018	China	38	CT	Association with gene expression profile	Manual, intratumoral	ROI	1	Individual textural features predicted gene modules	No
Wu *et al*[44], 2022	China	Training: 120. Validation: 52	CT	Ki-67 expression	Manual, intratumoral	VOI	2	AUC: 0.85 (training), 0.74 (validation)	Internal
Li *et al* [45], 2019	China	83	MRI	Ki-67 expression	Manual, intratumoral	ROI	2	Some features were associated, no model	No
Ye *et al* [47], 2019	China	89	MRI	Ki-67 expression	Manual, intratumoral	VOI	2	C-index: 0.878	No
Fan *et al*[46], 2021	China	Training: 103. Validation: 48	MRI	Ki-67 expression	Manual, intratumoral	VOI	2	AUC: 0.88 (training), 0.80 (validation)	Internal
Hu *et al* [48], 2022	China	Training: 87. Validation: 21	MRI	Ki-67 expression	Manual, intratumoral	ROI	1	AUC: 0.90 (training), 0.83 (validation)	Internal
Wang *et al* [50], 2019	China	78	MRI	CK19 positivity	Manual, intra- and peritumoral	ROI	1	AUC: 0.76	No
Chen *et al*[51], 2021	China	Training: 102. Validation: 19	MRI	CK19 positivity	Manual, intratumoral	ROI	2	AUC: 0.82 (training), 0.78 (external validation)	Internal and external
Yang *et al*[52], 2021	China (multicenter)	Training: 143. Validation: 75	MRI	CK19 positivity	Manual, intratumoral	ROI	2	AUC: 0.85 (training), 0.79 (external validation)	Internal and external
Wu *et al*[55], 2019	China	63	CT	P53 mutation status	Manual, intratumoral	ROI	2	AUC: 0.62-0.79	No
Li *et al* [99], 2022	China	92	MRI	Gene signatures associated with disease recurrence	Manual, intratumoral	ROI	2	MRI radiomics features could help quantify GOLM1, SETD7, and RND1 expression levels	Internal
Liao *et al*[56], 2022	China	Training: 86. Validation: 46	CT	Somatic mutations of the PI3K signaling pathway	Manual, intratumoral and peritumoral	VOI	2	AUC: 0.74 (training), 0.73 (external validation)	Internal and external
Che *et al*[60], 2022	China	Training: 69. Validation: 30	CT	β-arrestin1 phosphorylation	Manual, intratumoral	ROI	1	AUC: 0.89 (training), 0.74 (validation)	Internal

AUC: Area under the curve; CT: Computed tomography; MRI: Magnetic resonance imaging; ROI: Region of interest; VOI: Volume of interest; CK19: Cytokeratin 19; PI3K: P53, and phosphatidylinositol-3 kinase.

and decision cure analysis. The optimal model combining the arterial phase radiomic score and serum alpha-fetoprotein (AFP) levels showed high AUCs (AUC of 0.922 and 0.863 in the training and validation cohorts, respectively) for the preoperative Ki-67 expression prediction. In yet another study using Gd-EOB-DTPA-enhanced MRI, Ye *et al*[47] showed that the nomogram combining the texture signature (using the segmentation of the whole lesion) and clinical factors demonstrated a high discrimination ability (C-index of 0.936) for predicting Ki-67 group (high *vs* low). Finally, Hu *et al*[48] explored the added value of viscoelasticity measured by magnetic resonance elastography to predict Ki-67 expression, showing that shear wave speed and phase angle significantly improved the performance of the radiomic model.

CK19 expression: CK19 expression is associated with aggressive tumor behavior, resistance to therapy, and poor outcomes including worse overall survival and recurrence[49]. To date, three studies have focused on developing radiomic models to predict CK19 expression[50-52], all using MRI. Wang *et al*[50] showed that their texture model independently predicted CK19-positive HCC cases and improved the diagnostic performance of AFP level ≥ 400 ng/mL and arterial rim enhancement. The two remaining studies developed a radiomics model based on Gd-EOB-DTPA-enhanced MRI, with external validation AUC varying from 0.78-0.79; of note, one of the studies was a multicenter study with over 250 patients [51,52].

P53, PI3K, and other genetic expression: P53 can be used as a tumor biomarker, since it plays an important role in the pathogenesis of HCC[53]. P53 mutation has also been suggested as a feasible target for antitumor therapy[54]. Wu *et al*[55] demonstrated a direct relationship between P53 mutations in patients with HCC and the gray-level co-occurrence matrix on CT. PI3K signaling is a key pathway regulating HCC aggressiveness and is associated with response to sorafenib. Liao *et al*[56] developed a CT-based radiomics model that yielded an AUC of 0.73 in the external validation set for prediction of PI3K status.

The phosphorylation status of β-arrestin1 is associated with sorafenib resistance[57-59]. Che *et al*[60] developed a model combining a CT-based radiomics score with clinico-radiological risk factors which yielded an AUC of 0.898 in predicting β-arrestin1 phosphorylation, and the predicted β-arrestin1 phosphorylation was in turn significantly associated with overall survival in both the training and validation cohorts ($P < 0.05$).

Prediction of recurrence, treatment response, and liver failure

Tumor recurrence, liver failure and treatment response rates are major concerns during HCC treatment. Radiomics has emerged as a promising tool to predict recurrence and treatment response beyond the current predictive criteria[61,62]. Table 3 summarizes the studies to date that have evaluated the use of radiomic models to predict recurrence and treatment response. Most of these studies were single-center studies performed in China, with only a few studies incorporating external validation[63,64]. Segmentation strategies were predominantly manual strategies, including manual segmentation of the tumor region or area of interest, with only a few studies involving the segmentation of the peritumoral liver parenchyma[63,65-67]. Overall, the radiomic models yielded an AUC between 0.59 and 0.94 (see Table 3).

Of the studies evaluating the use of radiomics to predict recurrence, most involved the prediction of recurrence after surgical resection on CT or MRI, demonstrating a validation AUC between 0.59 and 0.84 (Table 3). Zhou *et al*[68] demonstrated that combining the radiomic signature with conventional preoperative variables significantly improved clinical model accuracy in early recurrence prediction (AUC of 0.84). Ji *et al*[64] developed and externally validated a radiomic model with better prognostic ability (C index ≥ 0.77, AUC of 0.78), lower prediction error (Brier score ≤ 0.14), and better clinical use compared with other staging systems and models. A few other studies evaluating the use of radiomics to predict recurrence involved the prediction of recurrence after liver transplant[69], transarterial chemoembolization (TACE)[67,70], and radiofrequency ablation (RFA)[71], demonstrating a validation AUC between 0.71 and 0.82.

Of the studies evaluating the use of radiomics to predict treatment response, a few involved the prediction of treatment response post-TACE[63,72,73]. In Canada, Ivanics *et al*[73] developed a CT-based radiomic model and achieved an AUC of 0.87 on the internal validation set. A large multi-center Chinese study by Chen *et al*[63] evaluating treatment response after TACE performed semi-automatic segmentation of the tumor and of the peritumoral region on contrast-enhanced CT in 585 patients, and the validation AUC was 0.90. One small study by Horvat *et al*[74] assessed treatment response after RFA using tumor 3D volumes of interest on MRI, yielding an AUC of 0.76 for the radiomics model, although the model lacked validation. Finally, two studies from China evaluated the use of radiomics to predict liver failure after surgical resection[75,76].

Prediction of survival

Table 4 summarizes the studies to date that have evaluated the use of radiomics to predict survival in patients with HCC. Four studies evaluated the use of CT-based radiomics to predict survival after hepatic resection, demonstrating an AUC between 0.71 and 0.81, with two of the four studies performing internal validation[39,77-79]. A few other studies evaluated the use of radiomics to predict survival after TACE[80], TARE[81], and RFA[82], all without validation.

Of the studies that involved the prediction of survival after hepatic resection, Xu *et al*[39] had the largest sample size. In their study, a risk model integrating clinico-radiological factors and a high CT-based radiomic score was independently associated with long-term mortality and disease-specific recurrence. Kim *et al*[80] evaluated the use of CT-based radiomics in survival prediction in patients after TACE. They demonstrated a combined model integrating radiomic features and clinical data (HCC size, Child-Pugh score and AFP) outperformed the clinical sore model or the radiomic score model. Petukhova-Greenstein *et al*[82] found that a higher MRI-based radiomic signature based on nodular and

Table 3 Summary of the studies that assessed radiomics to predict recurrence and treatment response in patient with hepatocellular cholangiocarcinoma who underwent surgery, liver transplantation or locoregional treatment

Ref.	Country	*n*	Imaging modality	Endpoint	Treatment type	Segmentation	ROI/VOI	No. of readers	Main results	Validation
Hui *et al* [100], 2018	Singapore	50	MRI	Recurrence	Hepatic resection	Manual, intratumoral	ROI	3	AUC: 0.78-0.84	None
Kim *et al* [65], 2019	South Korea	Training: 128. Validation: 39	MRI	Recurrence	Hepatic resection	Semiautomatic, intra- and peritumoral	VOI	2	C-index: 0.716	Internal
Zhao *et al*[101], 2021	China	Training: 78. Validation: 35	MRI	Recurrence	Hepatic resection	Manual, intratumoral	VOI	2	AUC: 0.83 (training), 0.77 (validation)	Internal
Zhou *et al*[68], 2017	China	215	CT	Recurrence	Hepatic resection	Manual, intratumoral	ROI	2	AUC: 0.84 (combined model)	None
Ji *et al* [64], 2020	China	Internal: 177. External: 118	CT	Recurrence	Hepatic resection	Manual, intratumoral	VOI	1	AUC: 0.77 (internal), 0.78 (external)	External
Guo *et al* [69], 2019	China	Training: 93. Validation: 40	CT	Recurrence	Liver transplant	Semiautomatic, intratumoral	ROI	1	AUC: 0.79 (training), 0.79 (validation)	Internal
Shan *et al*[66], 2019	China	Training: 109. Validation: 47	CT	Recurrence	Hepatic resection or ablation	Manual, intra- and peritumoral	ROI	2	AUC: 0.80 (training), 0.79 (validation)	Internal
Zheng *et al*[79], 2018	China	Training: 212. Validation: 107	CT	Recurrence and survival	Hepatic resection	Manual, intratumoral	ROI	2	AUC: 0.64 (training), 0.59 (validation)	Internal
Song *et al*[67], 2020	China	Training: 110. Validation: 74	MRI	Recurrence	TACE	Semiautomatic, intra- and peritumoral	VOI	2	C-index: 0.82	Internal
Lv *et al* [71], 2021	China	Training: 40. Validation: 18	MRI	Recurrence	RFA	Semiautomatic, intratumoral	VOI	2	AUC: 0.94 (training), 0.82 (validation)	Internal
Sun *et al* [70], 2020	China	Training: 67. Validation: 17	MRI	Recurrence	TACE	Manual (intratumoral)	VOI	2	AUC: 0.71-0.79	Internal
Cai *et al* [75], 2019	China	Training: 80. Validation: 32	CT	Liver failure	Hepatic resection	Semiautomatic, intratumoral	ROI	2	AUC: 0.82 (training), 0.76 (validation)	Internal
Zhu *et al* [76], 2020	China	101	MRI	Liver failure	Hepatic resection	Manual, entire liver	ROI	2	AUC: 0.81-0.89	None
Ivanics *et al*[73], 2021	Canada	88	CT	Treatment response	TACE	Manual, intratumoral	VOI	1	AUC: 0.70-0.87	None
Kong *et al*[72], 2021	China	Training: 69. Validation: 30	MRI	Treatment response	TACE	Manual, intratumoral	VOI	2	AUC: 0.81 (training), 0.87 (validation)	Internal
Chen *et al*[63], 2021	China	Training: 355. Internal: 118. External: 122	CT	Treatment response	TACE	Semiautomatic, intra- and peritumoral	ROI	2	AUC: 0.94 (internal), 0.90 (external)	Internal and external
Horvat *et al*[74], 2021	Brazil	34	MRI	Treatment response	RFA	Manual, intratumoral	VOI	1	AUC: 0.76	None

AUC: Area under the curve; CT: Computed tomography; MRI: Magnetic resonance imaging; RFA: Radiofrequency ablation; ROI: Region of interest; TACE: Transarterial chemoembolization; VOI: Volume of interest.

perinodular radiomic features predicted poorer survival after RFA. A study evaluated the survival prediction after TARE, using 18-fuoro-deoxyglucose PET-based radiomics[81]. They observed that whole-liver radiomics textural features were an independent negative predictor of survival.

Table 4 Summary of the studies that evaluated radiomics to predict survival in patients with hepatocellular cholangiocarcinoma

Ref.	Country	*n*	Imaging modality	Endpoint	Treatment type	Segmentation	ROI/VOI	No. of readers	Main results	Validation
Kiryu *et al* [77], 2017	Japan	122	CT	Survival	Hepatic resection	Manual, intra- and peritumoral	ROI	1	OS and DFS were significantly different between 2 rad score groups	None
Xu *et al*[39], 2019	China	Training: 350. Validation: 145	CT	Survival	Hepatic resection	Semiautomatic, intratumoral	VOI	3	AUC: 0.91 (training), 0.81 (validation)	Internal
Akai *et al* [78], 2018	Japan	127	CT	Survival	Hepatic resection	Manual, intrat-umoral	ROI	1	OS and DFS were significantly different between 2 rad score groups	None
Kim *et al* [80], 2018	South Korea	88	CT	Survival	TACE	Manual, intrat-umoral	ROI	1	Combined clinical and radiomics score was a better predictor of survival	None
Blanc-Durand *et al* [81], 2018	Switzerland	47	^{18}F-FDG PET-CT	Survival	TARE	Semiautomatic, whole liver	VOI	N/A	PFS-Rad Score and OS-Rad Score were independent negative predictors	None
Petukhova-Greenstein *et al*[82], 2022	United States	65	MRI	Survival	RFA	Semiautomatic, intra- and peritumoral	VOI	2	OS was significantly different between 2 rad score groups	None
Zheng *et al* [79], 2018	China	Training: 212. Validation: 107	CT	Survival	Hepatic resection	Manual, intrat-umoral	ROI	2	AUC: 0.71 (training and validation)	Internal

AUC: Area under the curve; CT: Computed tomography; DFS: Disease-fee survival; MRI: Magnetic resonance imaging; OS: Overall survival; PFS: Progression-free survival; RFA: Radiofrequency ablation; ROI: Region of interest; TACE: Transarterial chemoembolization; TARE: Transarterial radioembolization; VOI: Volume of interest; PET: Positron emission tomography.

Furthermore, radiomic scoring system did not differ after stratification by tumor size and Barcelona Clinic Liver Cancer staging.

Other applications of radiomics in HCC

Immunotherapy represents a paradigm shift in the management of patients with advanced HCC. Preoperatively assessing the immune status can assist the multidisciplinary team to identify which patients are suitable for immunotherapy, potentially improving treatment efficiency and overall survival rate. A few studies have evaluated the use of radiomics to predict programmed cell death ligand 1 (PD-L1) expression[83], CD8+ T cell infiltration[84], immunoscore[85,86], and anti-PD-1 treatment efficacy[87] in patients with HCC, with none of them performing external validation. Tian *et al*[83] were the first group to explore the efficacy of MRI-based radiomics to predict PD-L1 status. They proposed a model integrating radiomic and DL features for the quick and accurate assessment of PD-L1 expression levels in HCC patients before immune checkpoint inhibitor therapy which yielded an AUC of 0.897. Chen *et al*[85] demonstrated in 207 patients that radiomic features including those from the peritumoural region were associated with a validated "immunoscore". This score characterizes the tumor infiltrating lymphocyte population and theoretically reflects the immune phenotype of the tumor microenvironment.

RADIOMICS REPRODUCIBILITY IN HCC

Reproducibility refers to variations of the same patient across different imaging scenarios (*e.g.*, scanner or imaging parameters), while repeatability refers to variations of the same patient using the same imaging protocol. Table 5 summarizes the 13 studies to date that have studied the reproducibility of radiomics in HCC patients. Most of these studies were conducted in China (8/13; 62%). Seven were performed using CT (54%), 5 using MRI (38%), and 1 using both CT scan and MRI (8%). Different software programs were used for segmentation and feature extraction. Most studies adopted manual segmentation (11/13; 85%), and most evaluated first- and second-order features, with a few including shape and higher-order features. In addition to intra and inter-reader reproducibility, some also assessed the repeatability of radiomic features obtained through two separate exams from the same scanner, different scanners from different vendors and centers, 3D *vs* 2D segmentation, different contrast imaging phases, injection rates and pixel resolutions on contrast-enhanced CT, and different *b*-values on diffusion-weighted imaging on MRI.

Of note, one study showed that intra-reader tumoral and peritumoral reproducibility were greatest in MRI[88]. Another study showed that for test-retest (same MRI system, 2 different MRI exams), the intraclass correlation coefficient varied from 0.53-0.99 and the inter-platform reproducibility (MRI systems from 2 different vendors) varied from 0.58-0.99[89]. Regarding different contrast phases, Ibrahim *et al*[90] showed that 25% of extracted features had a concordance correlation coefficient (CCC) > 0.9 across arterial and portal venous phases. Perrin *et al*[91] demonstrated that the number of reproducible features decreased with variations in contrast injection rate, pixel resolution, and scanner model.

FUTURE DIRECTIONS OF RADIOMICS IN HCC

Despite the increasing and encouraging results in the literature concerning radiomics in patients with HCC, there are challenges and limitations to be overcome before its clinical implementation, particularly related to reproducibility and repeatability, lesion segmentation, model overfitting, multidisciplinary acceptance, and multi-modal data integration[23].

Patient selection, imaging data, segmentation strategy, image processing, feature selection, and computational processing are some factors that may affect the reproducibility and repeatability. Transparent patient accrual, data normalization, standard image manipulation, and feature extraction data are some strategies that may improve these challenges. Additionally, multi-center studies are recommended to increase reproducibility of the results.

Overfitting occurs when the model performs better in the training set with limited generalization of the results. The main factors contributing to overfitting are the number of included features being higher than the number of events and overoptimistic feature selection. Multiple strategies can be implemented to decrease overfitting, such as increasing the number of patients and events, using regularization methods, and including external validation cohorts. Multidisciplinary acceptance may improve with clear methods and a close relationship between radiologists, surgeons, oncologists, statistician, and data scientists to improve the interpretability of the results and to make way for clinical translation.

Multi-omics data integration is an additional step to improve the clinical acceptance of radiomics. Radiomics requires a multistep workflow process using different software and expertise; technological investments to create integrated and user-friendly tools are necessary to facilitate its widespread use in clinical practice. Finally, segmentation is a time-consuming process, susceptible to intra and inter-observer variability. Automatic and semi-automatic segmentations are required, particularly using DL strategies to facilitate this crucial step.

Additionally, some heterogeneity related to patients with HCC should be take into consideration. Since pathological confirmation is not always performed, the definition of clear and reproducible endpoints, like the LI-RADS criteria, are relevant strategies. Combined data integrating imaging and clinical variables are important to address the issue that patients with HCC are also dealing with systemic consequences related to cirrhosis.

CONCLUSION

Radiomics is an evolving computer-assisted tool with the potential to improve the multidisciplinary management of patients with HCC and to provide personalized treatment optimizing the available resources. Multiple studies have evaluated the use of radiomics in HCC with promising applications, including the prediction of pre-surgical histology, genetic signature, recurrence, and treatment response, as well as survival rates. Although promising, several challenges need to be overcome before radiomics can achieve clinical translation, including workflow optimization, model validation in multi-center studies, and the development of integrated models to facilitate clinical use and acceptance.

Table 5 Summary of the studies that assessed reproducibility of hepatocellular cholangiocarcinoma textural features

Ref.	Country	*n*	Imaging modality	Segmentation	Segmentation software	ROI/VOI	No. of readers	Intra-reader reproducibility	Inter-reader reproducibility	Other reproducibility
Duan *et al* [88], 2022	China	19	CT, MRI	Manual, intra- and peritumoral	3D-Slicer	ROI	2 (1 radiologist and 1 radiation oncologist)	Features with ICC ≥ 0.75 in both tumoral and peritumoral tissue greatest in MR	Features with ICC ≥ 0.75 in both tumoral and peritumoral tissue greatest in MR	N/A
Zhang *et al* [102], 2022	China	90 (31 HCC)	MRI	Manual, intratumoral	ITK-SNAP	ROI and VOI	2 radiologists	N/A	ICC > 0.8 used	N/A
Carbonell *et al*[89], 2022	United States	55 (16 HCC)	MRI	Manual, intratumoral and liver parenchyma	Olea sphere 3.0, Olea Medical	ROI for normal liver, VOI for HCC	2 radiologists	N/A	CCC: 0.80-0.99	For test-retest (same MRI system, 2 different MRI exams): ICC: 0.53-0.99; and in liver parenchyma: ICC: 0.53-0.73. For inter-platform reproducibility (MRI systems from 2 different vendors): CCC: 0.58-0.99
Park *et al* [103], 2022	South Korea	249	CT	Manual followed by automatic segmentation, intratumoral	MEDIP PRO	ROI and VOI	1 radiologist	For VOI: Manual: ICC 0.594-0.998 for FO, 0.764-0.997 for shape, and 0.190-0.926 for SO; DL-AS: ICC > 0.75 for all. For ROI: Manual: 0.698-0.997 for FO, 0.556-0.997 for shape, and 0.341-0.935 for SO; DL-AS ICC > 0.75 for all	N/A	
Haniff *et al* [104], 2021	Malaysia	30	MRI	Manual and semi-automatic, intratumoral	3D-Slicer	VOI	Manual: 4 readers. Semi-automatic: 2 readers	N/A	Manual segmentation: ICC 0.897. Semi-automatic segmentation: ICC 0.952	NA
Ibrahim *et al*[90], 2021	Germany	61 patients, 104 lesions	CT	Manual, intratumoral	MIM software	ROI	1 nonradiologist revised by radiologist	N/A	N/A	Across different contrast imaging phases: 25% of extracted features had CCC > 0.9 across arterial and portal venous phases
Hu *et al* [105], 2021	China	30	CT	Manual, intratumoral	MaZda software	ROI	2 radiologists	ICC > 0.7	ICC > 0.7	N/A
Mao *et al* [32], 2020	China	30	CT	Manual, intratumoral	ITK-SNAP	ROI	2 radiologists	N/A	ICC ≥ 0.8	N/A
Hu *et al* [106], 2020	China	50	CT	Semi-automatic, peritumoral	Not mentioned	ROI	2 radiologists	N/A	ICC > 0.6	N/A
Qiu *et al* [107], 2019	China	26	CT	Manual and semi-automatic, intratumoral	GrowCut and GraphCut	ROI	Manual: 5 radiation oncologists. Semi-automatic: 2 radiation oncologists	N/A	ICC ≥ 0.75 in 69% of features extracted from manual segmentation, 73% from GraphCut, and 79% from GrowCut	Across different centers: Poor reproducibility of CT-based peritumoral-radiomics model
Zhang *et al* [108], 2019	China	46 (34 HCC)	MRI	Manual, intratumoral	MIM software	VOI	1 radiologist	N/A	N/A	Across different *b*-values: radiomic features extracted from *b* = 0, 20, 50,

										100, 200 s/mm^2 and b = 1000 s/mm^2 and nearby b-values DWIs showed a high reproducibility (ICC ≥ 0.8)
Feng *et al* [40], 2019	China	160 (110)	MRI	Manual, intra- and peritumoral	ITK-SNAP	VOI	3 radiologists	85% ICC ≥ 0.8	82% ICC ≥ 0.8	N/A
Perrin *et al* [91], 2018	United States	38 (6 HCC)	CT	Semi-automatic, intratumoral and liver parenchyma	Scout Liver	VOI	1 research fellow under supervision of radiologist	N/A	N/A	Across different contrast injection rates, pixel resolutions, and scanner models: Number of reproducible radiomic features (CCC > 0.9) decreased with variations in contrast injection rate, pixel resolution, and scanner model

CT: Computed tomography; MRI: Magnetic resonance imaging; ROI: Region of interest; VOI: Volume of interest; TACE: Transarterial chemoembolization; ICC: Intraclass correlation coefficient; DWI: Diffusion-weighted imaging; CCC: Concordance correlation coefficient; HCC: Hepatocellular carcinoma; N/A: Not applicable; FO: First order; SO: Second order; DL-AS: Deep learning-based auto-segmentation.

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Country/Territory of origin: Brazil

ORCID number: Joao Miranda 0000-0003-1273-3108; Gilton Marques Fonseca 0000-0002-7260-0799; Paulo Herman 0000-0003-2859-5846.

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L-Editor: A
P-Editor: Wang JJ

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REVIEW

Evolution of care in cirrhosis: Preventing hepatic decompensation through pharmacotherapy

Seohyuk Lee, Saad Saffo

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Seohyuk Lee, Saad Saffo, Department of Medicine, Section of Digestive Diseases, Yale School of Medicine, New Haven, CT 06520-8019, United States

Corresponding author: Saad Saffo, MD, Academic Fellow, Department of Medicine, Section of Digestive Diseases, Yale School of Medicine, 333 Cedar Street, 1080 LMP, New Haven, CT 06520-8019, United States. saad.saffo@yale.edu

Abstract

Cirrhosis is a leading cause of morbidity and mortality, impacting more than 120 million people worldwide. Although geographic differences exist, etiologic factors such as alcohol use disorder, chronic viral hepatitis infections, and non-alcoholic fatty liver disease are prevalent in nearly every region. Historically, significant effort has been devoted to modifying these risks to prevent disease progression. Nevertheless, more than 11% of patients with compensated cirrhosis experience hepatic decompensation each year. This transition signifies the most important prognostic factor in the natural history of the disease, corresponding to a decline in median survival to below 2 years. Over the past decade, the need for pharmacotherapies aimed at reducing the risk for hepatic decompensation has been emphasized, and non-selective beta-blockers have emerged as the most effective option to date. However, a critical therapeutic gap still exists, and additional therapies have been proposed, including statins, rifaximin, and sodium-glucose cotransporter-2 inhibitors. Based on the results of innovative retrospective analyses and small-scale prospective trials, these pharmacotherapies represent promising options, but further studies, including randomized controlled trials, are necessary before they can be incorporated into clinical use. This report highlights the potential impact of these agents and others in preventing hepatic decompensation and discusses how this paradigm shift may pave the way for guideline-directed medical therapy in cirrhosis.

Key Words: Cirrhosis; Hepatic decompensation; Beta-blockers; Statins; Sodium-glucose cotransporter-2 inhibitors; Rifaximin

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Core Tip: Hepatic decompensation is the most important clinical predictor of morbidity and mortality among patients with cirrhosis. New pharmacotherapies aimed at preventing hepatic decompensation in high-risk patients are emerging, augmenting traditional management strategies. These treatments represent safe, accessible, and effective options that may improve quality of life and prolong transplant-free survival, regardless of the etiologic factors involved.

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INTRODUCTION

Cirrhosis reflects the end stage of all chronic liver diseases. It impacts more than 120 million people globally, largely in the context of common risk factors such as alcohol use disorder, chronic viral hepatitis, and non-alcoholic fatty liver disease (NAFLD)[1]. It represents the 8th leading cause of mortality in the United States and 13th in the world, with the number of attributable deaths worldwide having increased by approximately 50% between 1990 and 2013[2]. Regardless of etiology, the most important prognostic factor for survival is the presence or absence of hepatic decompensation, which includes complications such as ascites, variceal hemorrhage, hepatic encephalopathy, and jaundice[3,4]. Although patients with cirrhosis may remain compensated for extended periods of time, especially if their underlying risk factors are mitigated, approximately 11% of those with compensated disease experience new decompensations each year[5], and the progression from compensated to decompensated cirrhosis is associated with a decline in median survival from 12 years to less than 2 years[6]. Among individuals with compensated disease, the 10-year risks of developing ascites, gastrointestinal hemorrhage, hepatic encephalopathy, and jaundice are 40%, 25%, 28%, and 33%, respectively[6].

Beyond its burden on patient health and quality of life, cirrhosis also represents a critical healthcare challenge. According to the Global Burden of Disease Study 2017[1], there are 112 million and 10.6 million cases of compensated and decompensated cirrhosis worldwide, respectively. In the United States, this is associated with significant healthcare costs, including approximately $2.5 billion annually for hospitalization and treatment and $10.6 billion annually for losses in work productivity and health-related quality of life[7]. These costs are disproportionately borne in the management of complications among those with decompensated disease. Despite the significant health and socioeconomic burdens, it is only recently that management strategies have pivoted from focusing primarily on risk factor modification and the treatment of complications towards the prevention of hepatic decompensation in high-risk patients. Unlike the management of other common chronic diseases such as congestive heart failure, in which the implementation of guideline-directed medical therapy has resulted in significant reductions in morbidity and mortality, the long-term management of cirrhosis has been historically limited by a lack of robust chemoprevention[8,9]. In this report, we discuss the management of cirrhosis, focusing on pharmacotherapies aimed at preventing hepatic decompensation.

TRADITIONAL TREATMENT STRATEGIES

Chronic hepatitis C virus (HCV) infection, NAFLD, and alcohol-related liver disease are the three leading causes of cirrhosis in the United States[10]. For each of these etiologies, a critical component of the long-term management involves risk factor modification. Among patients with chronic HCV infection and compensated disease, the advent of direct-acting antiviral agents represents a landmark achievement that has been shown to reduce the risk for liver-related complications[11-14]. Unfortunately, more than 40% of HCV infections are diagnosed after hepatic decompensation has already occurred[10], at which point antiviral treatment is less effective and associated with a higher risk for adverse events[15]. For those with NAFLD, a growing body of literature supports the efficacy of a multimodal approach which integrates dietary changes, weight reduction, and restoring insulin sensitivity[16]. Glucagon-like peptide 1 receptor agonists such as semaglutide have an evolving role in the treatment in of NAFLD, offering both weight reduction and possible attenuation of steatohepatitis, and a number of other drugs aimed at reducing fibrosis progression and the risk for complications are currently being evaluated in phase II and phase III clinical trials[17]. However, to date, disease-specific pharmacologic treatments have yet to be approved. The prevalence of NAFLD and its associated complications continue to increase[18] such that it is currently the second leading cause of cirrhosis among patients awaiting liver transplantation in the United States[14] and is expected to overtake HCV-

related cirrhosis as the most frequent indication for liver transplantation[19]. Finally, alcohol-related liver disease remains highly prevalent worldwide[20] with increasing cirrhosis-related mortality in regions with high alcohol consumption[21]. Abstaining from alcohol significantly improves survival among patients with cirrhosis, but pharmacologic options for alcohol use disorder remain limited, partially due to concerns about hepatotoxicity[22]. For patients with less common causes of chronic liver disease, risk factor modification may include therapies such as immunosuppression (autoimmune hepatitis), ursodeoxycholic acid (primary biliary cholangitis), and phlebotomy or chelation (hemochromatosis). Unfortunately, the impact of some of these treatments is generally diminished in the context of cirrhosis.

Once hepatic decompensation has occurred, treatment strategies may be implemented to address specific complications. This includes pharmacotherapy to eliminate ascites (diuretics), reduce the risk of variceal rupture [non-selective beta-blockers (NSBBs)], and prevent recurrent encephalopathy (lactulose and rifaximin)[23-25]. Among patients with refractory ascites or variceal hemorrhage, transjugular intrahepatic portosystemic shunting (TIPS) reduces the risk of further decompensation and may improve survival in a subset of patients[26-28]. Finally, liver transplantation is the only durable curative option for patients with decompensated cirrhosis. However, resource limitations and host factors restrict the use of TIPS and liver transplantation to a relatively small number of patients in resource-rich settings. Furthermore, despite advances in the care of decompensated cirrhosis, the 30-d mortality following hospital discharges for hepatic decompensation remains largely unchanged[29]. Thus, significant interest exists in preventing the progression to decompensated cirrhosis.

CURRENT THERAPIES TO PREVENT HEPATIC DECOMPENSATION

NSBBs are currently the only agents recommended for the long-term management of portal hypertension in patients with compensated cirrhosis[30]. Traditional NSBBs such as propranolol and nadolol inhibit β1 and β2 receptors, mitigating the hyperdynamic response and splanchnic arteriolar vasodilation that occur in the context of cirrhosis. Carvedilol has additional α1 inhibition that reduces intrahepatic vascular resistance[31,32]. Together, these hemodynamic effects attenuate portal hypertension, reducing the risk for hepatic decompensation. In addition, NSBBs impact the risk for liver-related complications through other mechanisms. For example, NSBBs reduce abnormal gastrointestinal permeability and bacterial translocation[33] and upregulate the phagocytic activity of monocytes and granulocytes after exposure to bacterial DNA[34]. As such, a recent prospective study demonstrated that NSBBs reduce the risk for bacterial infections [odds ratio = 0.46, 95% confidence interval (CI): 0.3-0.7; $P = 0.001$][35].

The landmark trial demonstrating the utility of NSBBs in preventing decompensation was the study on Beta-Blockers to Prevent Decompensation of Cirrhosis with Portal Hypertension (PREDESCI)[36]. In this multicenter, double-blind, randomized controlled trial (RCT), 201 patients with compensated cirrhosis and clinically-significant portal hypertension (CSPH) without high-risk varices were randomized to receive NSBBs (propranolol up to 160 mg twice daily or carvedilol up to 25 mg daily) or placebo. Over a median follow-up of 37 mo, the risk of hepatic decompensation, including ascites, variceal hemorrhage, or hepatic encephalopathy, was significantly lower among patients receiving NSBBs relative to those receiving placebo [hazard ratio (HR) = 0.51, 95%CI: 0.26-0.97; $P = 0.041$]. This difference was driven primarily by a reduction in ascites (HR = 0.42, 95%CI: 0.19-0.92; $P = 0.03$), although non-significant trends towards decreased progression to high-risk varices and improved survival were also observed. The number needed to treat for the prevention of a decompensation event over the follow-up period was 9, and the incidence of adverse events was similar between the treatment and placebo groups.

Prior to PREDESCI, other RCTs consistently demonstrated that propranolol and nadolol were effective in preventing variceal hemorrhage among patients with cirrhosis and large esophageal varies but without prior bleeding episodes. Pascal and Cales[37] found that patients receiving propranolol up to 320 mg daily were less likely to develop bleeding episodes compared to those in the placebo group (74% *vs* 39%; $P < 0.05$); Idéo *et al*[38] also observed significantly lower rates among those receiving nadolol up to 120 mg daily (94.4%) relative to those receiving placebo (70.2%). Other studies have reported similar risk reductions in initial[39,40] and subsequent[41] variceal bleeds, although one reported a significant difference only among patients who lacked ascites[42]. Given these data, NSBBs are currently recommended for the primary and secondary prophylaxis of variceal hemorrhage[37,41]. Notably, however, the Prevention of Esophageal Varices by Beta-Adrenergic Blockers trial observed no differences in the use of timolol up to 80 mg daily *vs* placebo in the development of varices among patients with compensated cirrhosis[43].

The potential benefits of carvedilol beyond those of traditional NSBBs have also been of great interest. Four studies have evaluated the impact of carvedilol in preventing hepatic decompensation or disease progression among patients with compensated cirrhosis and CSPH. A subgroup analysis of the PREDESCI trial[36] found a non-significant reduction in the risk for hepatic decompensation or death (HR = 0.39, 95%CI: 0.10-1.49; $P = 0.16$) and Bhardwaj *et al*[44] observed a significantly higher likelihood

of non-progression from small to large esophageal varices (79.4% *vs* 61.4%; P = 0.04). In comparing carvedilol to variceal band ligation, one study found carvedilol 12.5 mg daily to be associated with significantly lower rates of initial variceal hemorrhage (HR = 0.41, 95%CI: 0.19-0.96; P = 0.04) but similar rates of bleeding-related and overall mortality[45], while another reported comparable rates across all three outcomes with the same dosing[46]. Additionally, a recent meta-analysis of these four studies observed a significantly improved hazard ratio for decompensation among patients receiving carvedilol compared to control therapy (HR = 0.506, 95%CI: 0.289-0.887; P = 0.017)[47]. Finally, in comparison to propranolol, multiple studies, including PREDESCI, have suggested that carvedilol may be superior in reducing the hepatic venous pressure gradient[36,48]. However, it remains unclear whether this finding consistently translates into a difference in the risk for hepatic decompensation.

Although NSBBs have been extensively studied and represent the current treatment of choice for the prevention of hepatic decompensation, there is significant interest in identifying additional therapies for chemoprevention for a variety of reasons. First, most commonly, patients may have contraindications or intolerance to NSBBs. Common adverse effects include bradycardia, hypotension, and fatigue. Second, pharmacotherapy may be leveraged for pleiotropic benefits, and alternative therapies may provide an opportunity for enhanced personalized care. Finally, therapies may have additive or synergistic effects that may provide additional clinical benefit for high-risk patients. As such, a number of agents are currently under evaluation (Table 1).

EMERGING THERAPIES TO PREVENT HEPATIC DECOMPENSATION

Statins

These cholesterol-lowering drugs are a mainstay for the treatment of dyslipidemia and atherosclerotic cardiovascular disease but have diverse pleiotropic effects that may impact a wide range of other disease processes. Concerns regarding hepatotoxicity have historically limited their use among patients with chronic liver disease[49], but emerging evidence indicates that their anti-fibrotic, immunomodulatory, and antioxidant effects may attenuate portal hypertension and limit disease progression[50-53] without posing an excess safety risk among patients with compensated disease[54-56].

Several retrospective studies have evaluated the role of statins in preventing hepatic decompensation. In one case-control study of patients with predominantly early-stage cirrhosis, statin use was associated with a decreased risk of hepatic decompensation over 36 mo (HR = 0.58; P = 0.04)[57]. Similar findings have been reported in patients with cirrhosis due to chronic viral hepatitis; among statin users, the HR for hepatic decompensation was 0.39 (95%CI: 0.25-0.62)[58] for hepatitis B virus-related cirrhosis and 0.51 (95%CI: 0.29-0.93)[58] to 0.55 (95%CI: 0.39-0.77)[59] for HCV-related cirrhosis. A trend towards decreased hepatic decompensation was observed with among patients with alcohol-related cirrhosis (HR = 0.69, 95%CI: 0.45-1.07)[58]. A recent meta-analysis of these three observational studies found the pooled HR for hepatic decompensation to be 0.54 (95%CI: 0.46-0.65) with minimal heterogeneity (I^2 = 0%)[60]. A similar meta-analysis using these studies also demonstrated that the association between statin use and an improved decompensation was independent of cirrhosis etiology[61].

In light of the encouraging findings reported in observational studies, RCTs evaluating the impact of statins in preventing hepatic decompensation are currently in progress. In the United States, the Statins and Cirrhosis: Reducing Events of Decompensation trial is studying simvastatin at a dose of 40 mg daily (ClinicalTrials.gov, NCT03654053)[62]. In Denmark, the Statins for Prevention of Disease Progression and Hospitalization in Liver Cirrhosis trial is studying atorvastatin at doses of 10-20 mg daily (ClinicalTrials.gov, NCT04072601).

Rifaximin

Patients with cirrhosis can experience increased bacterial translocation secondary to elevated portal pressures, increased gastrointestinal permeability, and altered gut microbiota, thereby contributing to the inflammatory milieu. Rifaximin is a safe poorly-absorbed oral antibiotic with broad gut-selective antimicrobial activity against gram-positive and gram-negative bacteria. Beyond its impacts on the intestinal microbiome, it may also attenuate inflammation, decrease bacterial interactions with enterocytes, and improve intestinal epithelial integrity[63,64]. Furthermore, the combination of propranolol and rifaximin compared to propranolol alone is associated with a more significant reduction in portal pressure[65]. Although rifaximin is currently approved for the prevention of recurrent hepatic encephalopathy[66], it may also have a role in preventing other hepatic decompensations.

A number of studies have evaluated the association between rifaximin use and liver-related complications, demonstrating that rifaximin may reduce the risk of further decompensation in patients with decompensated cirrhosis[63]. However, little is known about its role in those with high-risk compensated disease. Most notably, in a *post-hoc* analysis of a RCT comparing rifaximin 550 mg twice daily to placebo, Flamm *et al*[67] demonstrated that rifaximin reduces the risk for further hepatic decompensation (HR = 0.41, 95%CI: 0.25-0.67; P < 0.001). This finding was corroborated by Zeng *et al*[68] in a prospective randomized open-labelled study. Currently, there are no ongoing trials investigating

Table 1 Chemoprevention for hepatic decompensation in cirrhosis: Current and emerging therapies

Agents	Mechanism of action	Primary benefits	Potential adverse effects	Other limitations	Supported by RCT
NSBBs	β1 and β2 blockade; α1 blockade (carvedilol)	Decreased portal pressure	Hypotension, bradycardia, fatigue	Dosing frequency (propranolol)	Yes
Statins	Inhibition of HMG-CoA reductase	Decreased inflammation and endothelial dysfunction	Myopathy, hepatitis, diabetes		Ongoing
Rifaximin	Broad-spectrum, gut-specific antibiotic	Reduced dysbiosis and bacterial translocation	Gastrointestinal upset	Cost	Included patients with prior decompensation
Anticoagulants	Inactivation of clotting factors	Reduced endothelial dysfunction	Hemorrhage	SQ injection (enoxaparin), dosing (warfarin)	Included patients with prior decompensation
ACE inhibitors	Inhibition of angiotensin II production	Decreased portal pressure[1]	Hypotension, AKI, electrolyte derangements, angioedema		No
ARBs	Inhibition of angiotensin II type 1 receptor	Decreased portal pressure	Hypotension, AKI, electrolyte derangements		No
MRAs	Inhibition of the aldosterone receptor in the distal nephron	Decreased portal pressure	Hypotension, AKI, electrolyte derangements	Gynecomastia (spirono-lactone)	Only in combination with NSBB
SGLT2 inhibitors	Inhibition of proximal tubule sodium-glucose cotransporter	Decreased portal pressure	Electrolyte derangements, mycotic genital infections	Cost, risk of ketoacidosis in AUD	No
Albumin	Anionic carrier protein with pleiotropic properties	Reduced inflammation; increased effective circulating volume	Volume overload	Cost, intravenous administration	Included patients with prior decompensation

[1]Not demonstrated in prior clinical studies.

ACE: Angiotensin converting enzyme; AKI: Acute kidney injury; ARB: Angiotensin receptor blocker; AUD: Alcohol use disorder; BP: Blood pressure; HMG-CoA: Hydroxy β-methylglutaryl-CoA; MRA: Mineralocorticoid receptor antagonist; NSBB: Non-selective beta-blocker; SGLT2: Sodium-glucose cotransporter 2; SQ: Subcutaneous; RCT: Randomized controlled trial.

the impact of rifaximin in patients with compensated disease, although the Simvastatin Plus Rifaximin in Decompensated Cirrhosis study is examining the role of statins and rifaximin among patients with pre-existing decompensated disease (ClinicalTrials.gov, NCT03780673). Historically, a critical barrier to the study and use of rifaximin has been cost.

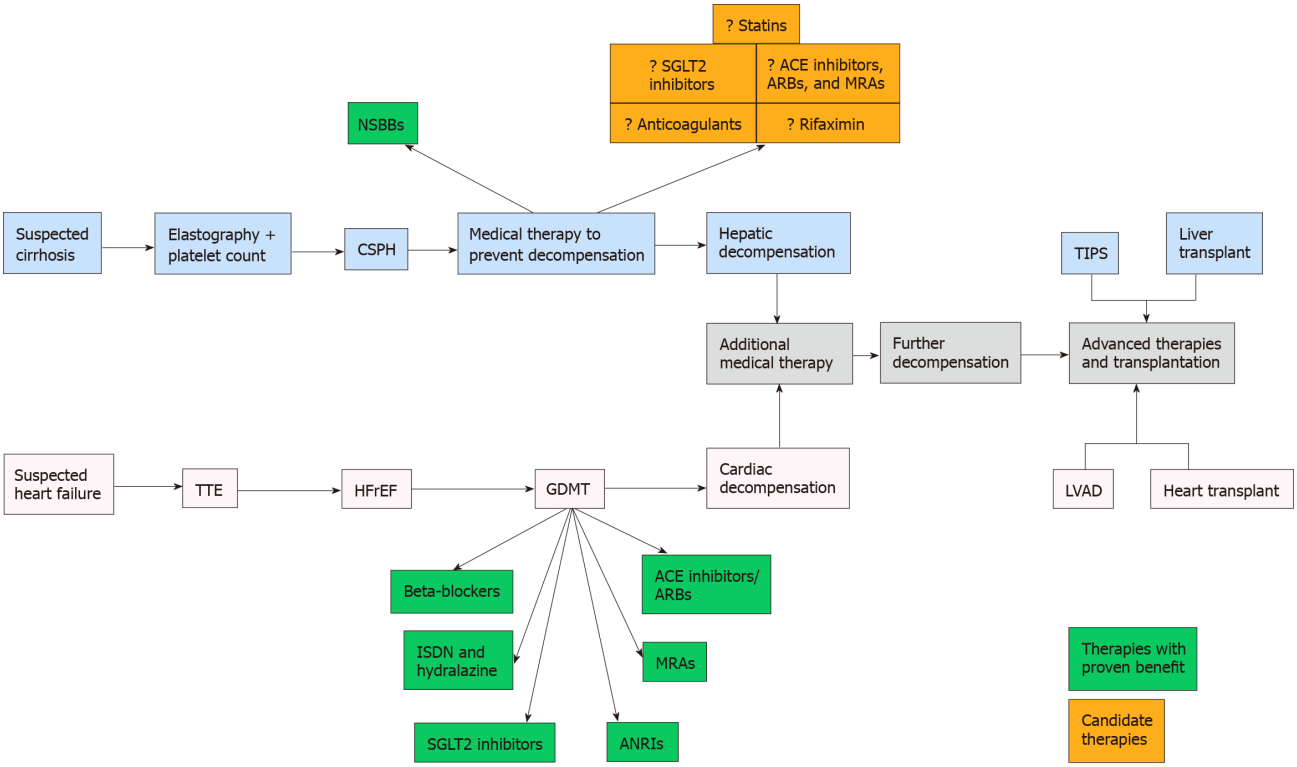
Anticoagulants

In light of the impaired synthesis of clotting factors and the presence of thrombocytopenia associated with cirrhosis, the risk of bleeding has historically been prioritized over thrombosis[69]. However, more recent evidence suggests that a new state of rebalanced hemostasis is achieved among those with stable cirrhosis in which decompensation can lead to increased risks of both hemorrhage and thrombosis, which in turn, can increase the risk for further decompensation[69]. Thrombin has been proposed to activate hepatic stellate cells and lead to upregulation of hepatic fibrosis, suggesting a potential role for anticoagulation in slowing cirrhosis disease progression[70].

Several studies have evaluated the efficacy and safety of anticoagulation in preventing or managing venous thrombotic events in patients with cirrhosis, but only two have specifically addressed its association with preventing decompensation[71-75]. In a RCT of patients with advanced cirrhosis randomized to receive enoxaparin 4000 IU/d or no treatment, decompensation was significantly less common among those receiving enoxaparin (11.7% *vs* 59.4%; $P < 0.0001$)[75]. Enoxaparin was independently associated with a reduced risk for hepatic decompensation (HR = 0.331, 95%CI: 0.148-0.741; $P = 0.007$). Notably, following cessation of enoxaparin receipt, rates of hepatic decompensation were similar between those in the control and treatment groups. In contrast, in a retrospective study of patients with cirrhosis and portal vein thrombosis, the 1-year probability of hepatic decompensation was not significantly different between patients who did or did not receive warfarin (15.6% *vs* 17.9%; $P = 0.847$)[72]. The ongoing CIRROXABAN phase III RCT (ClinicalTrials.gov, NCT02643212) aims to evaluate the effect of rivaroxaban in the development of decompensation among patients with cirrhosis.

Renin-angiotensin-aldosterone system antagonists

The renin-angiotensin-aldosterone system (RAAS) has a central role in the pathogenesis and progression of portal hypertension. Splanchnic and peripheral vasodilation in response to excess nitric



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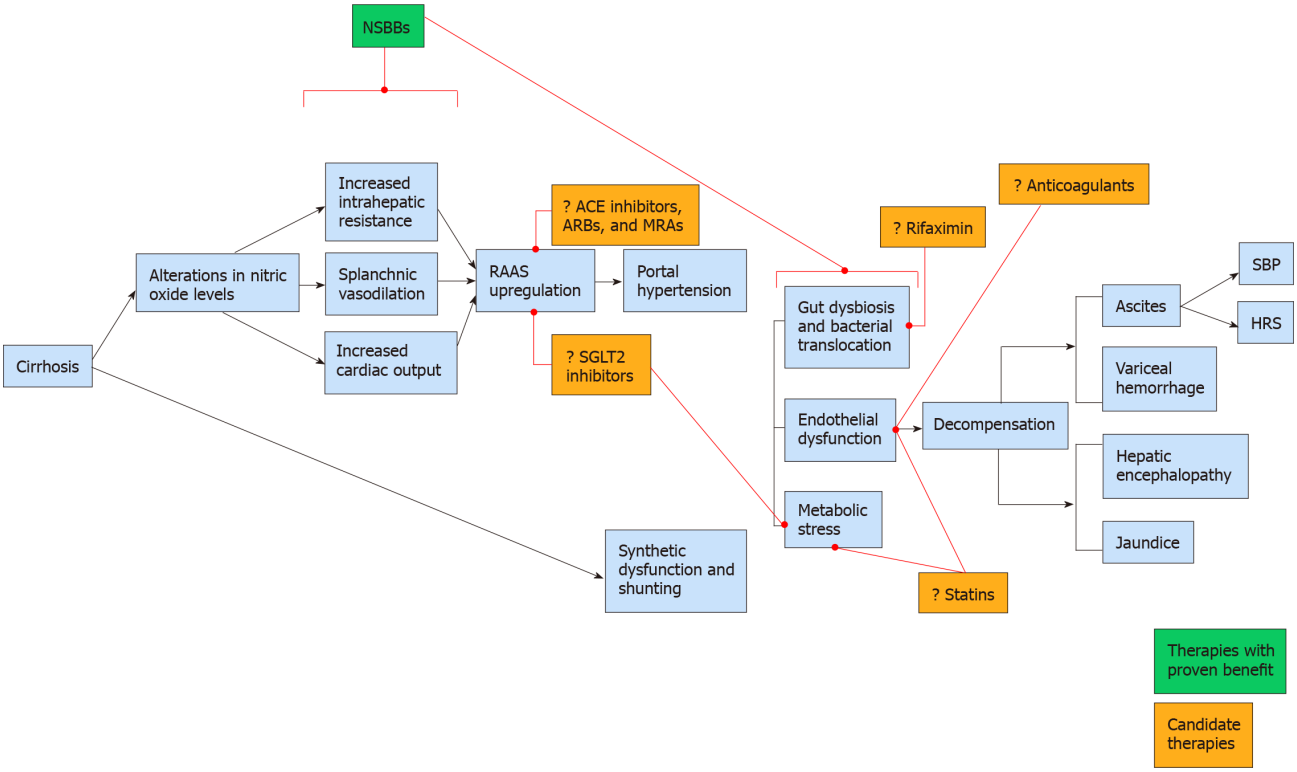
Figure 1 Management of cirrhosis in comparison to congestive heart failure. ACE: Angiotensin converting enzyme; ANRI: Angiotensin receptor-neprilysin inhibitor; ARB: Angiotensin receptor blocker; CSPH: Clinically-significant portal hypertension; GDMT: Guideline-directed medical therapy; HFrEF: Heart failure with reduced ejection fraction; LVAD: Left ventricular assist device; MRA: Mineralocorticoid receptor antagonist; NSBB: Non-selective beta-blocker; SGLT2: Sodium-glucose cotransporter 2; TIPS: Transjugular intrahepatic portosystemic shunting; TTE: Transthoracic echocardiogram.

oxide stimulate the renin-angiotensin-aldosterone and sympathetic nervous systems, triggering a number of mechanisms that further exacerbate portal hypertension. Mediators of these pathways have been directly linked to mortality in patients with cirrhosis[76,77]. Thus, a number of drugs which attenuate the RAAS have been evaluated with mixed results. These include angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), mineralocorticoid receptor antagonists such as spironolactone, and sodium-glucose cotransporter-2 (SGLT2) inhibitors.

Both ACE inhibitors and ARBs have been studied in portal hypertension, but their impact in preventing hepatic decompensation remains unclear. Current evidence suggests that ARBs may reduce portal pressures[78-84], but they have yet to be evaluated in RCTs. In contrast, ACE inhibitors have not been shown to reduce portal pressures[85], but a recent large retrospective analysis suggest that they may reduce the risk for liver-related complications in patients with NAFLD[86]. Regardless, while both agents may be safe and potentially beneficial in patients with early-stage disease, their side-effect profile may be deleterious among patients with CSPH.

Spironolactone is critical in the management of cirrhotic ascites. However, the drug has been investigated as an adjunctive agent to NSBBs in the prevention of variceal hemorrhage. A prospective trial demonstrated that the addition of spironolactone 100 mg daily to nadolol reduced the risk of a combined endpoint of variceal hemorrhage and ascites (39% *vs* 20%; $P < 0.04$)[87]. Further trials exploring the utility of spironolactone in preventing hepatic decompensation among compensated patients have not been pursued, and thus the agent is currently not recommended for chemoprevention.

Initially introduced as antidiabetic drugs, SGLT2 inhibitors have become mainstays in the management of cardiovascular disease and chronic kidney disease among diabetic and nondiabetic patients due to their broad pleiotropic effects[88-93]. By inhibiting sodium and glucose reabsorption in the proximal convoluted tubule, SGLT2 inhibitors restore tubuloglomerular feedback, attenuate overactivation of the RAAS and the sympathetic nervous system, and promote natriuresis, thereby overcoming key mechanisms that are also implicated in the pathogenesis of portal hypertension in cirrhosis[9]. Unlike ACE inhibitors, ARBs, and spironolactone, SGLT2 inhibitors have limited effects on systemic blood pressure and may be better tolerated among patients with CSPH. Although RCTs in cirrhosis are currently lacking, retrospective studies suggest that these agents, namely empagliflozin, are likely safe and warrant further investigation[94-98].



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Figure 2 Therapeutic targets in the prevention of hepatic decompensation. ACE: Angiotensin converting enzyme; ARB: Angiotensin receptor blocker; HRS: Hepatorenal syndrome; MRA: Mineralocorticoid receptor antagonist; NSBB: Non-selective beta-blocker; RAAS: Renin-angiotensin-aldosterone system; SBP: Spontaneous bacterial peritonitis; SGLT2: Sodium-glucose cotransporter 2.

Albumin

Albumin has versatile anti-inflammatory and plasma expansion properties that may also mitigate mechanisms implicated in hepatic decompensation. To our knowledge, no study has investigated the role of albumin in preventing hepatic decompensation among patients with compensated disease. However, the Human Albumin for the Treatment of Ascites in Patients with Hepatic Cirrhosis study assessed the impact of human albumin in patients with uncomplicated ascites[99]. Although the primary outcome was mortality, secondary analyses demonstrated that weekly 40-gram albumin infusions reduced the risk for refractory ascites, spontaneous bacterial peritonitis, hepatorenal syndrome, and hepatic encephalopathy in comparison to standard medical therapy alone. Notably, the study was limited by the fact that the treatment group received more frequent outpatient evaluations, allowing providers to address potential impending complications in a more proactive manner. Because of accessibility and cost limitations, in addition to other factors that restrict widespread use, it is unlikely that albumin will receive significant consideration as a potential therapeutic option in patients with compensated cirrhosis.

Other candidate pharmacotherapies

Numerous other agents hypothesized to decrease the risk of hepatic decompensation have been evaluated. Despite early studies suggesting indirect benefits, there is a paucity of consistent, rigorous clinical evidence demonstrating a direct role for nitrates[100-103], endothelin-A antagonists[104,105], farnesoid X receptor agonists[106], phosphodiesterase-5 inhibitors[107-111], serelaxin[112], sorafenib [113-115], taurine[116], and thalidomide[117] in the prevention of hepatic decompensation. Furthermore, it has been postulated that aspirin and metformin could be attractive targets. However, at this time, a number of prospective and retrospective studies have only demonstrated that these agents may reduce the risk of hepatocellular carcinoma without necessarily impacting the risk for hepatic decompensation[118-120].

A NEW PARADIGM: CHEMOPREVENTION IN THE MODERN ERA

Until PREDESCI validated the use of NSBBs for chemoprevention, the pharmacologic management of cirrhosis had experienced little progress over the preceding half century. In contrast, over the same period of time, incremental advances in the medical management of systolic heart failure, a condition

that mechanistically mimics portal hypertension, led to the widespread implementation of guideline-directed medical therapy, which significantly improved survival (Figure 1). In the past, the lack of non-invasive tools to identify high-risk patients hindered the development and application of chemoprevention in cirrhosis. However, with the advent of elastography and the validation of clinical prediction tools that incorporate laboratory markers such as platelet count, clinicians are now able to risk-stratify patients more efficiently, paving the way for more robust medical management. Based on the findings of the ANTICIPATE study and the guidelines proposed in the Baveno VII workshop[121, 122], patients who have a high likelihood for CSPH based on a combination of liver stiffness measurements and platelet count should be initiated on appropriate chemoprevention with a NSBB in the absence of contraindications to therapy. As additional agents for chemoprevention are evaluated over the coming years, a diverse multi-targeted strategy (Figure 2) may become feasible, mimicking the approach currently utilized for congestive heart failure. Although there are a number of candidate drugs, statins, rifaximin, and SGLT2 inhibitors currently offer the most promise, combining potential efficacy with other important considerations such as safety, accessibility, and systemic benefits.

CONCLUSION

The development of hepatic decompensation represents the most important prognostic factor in the natural history of cirrhosis. Treatments that mitigate this risk have an important role in the management of patients with CSPH. In light of the development of robust non-invasive tools that allow for the timely and accurate risk stratification of patients with cirrhosis, the application of chemoprevention is becoming increasingly feasible. NSBBs are currently the mainstays of treatment in this regard, but emerging therapies such as statins, rifaximin, and SGLT2 inhibitors, may offer hope for personalized multimodal strategies in the future. This paradigm shift may ultimately reduce liver-related morbidity and mortality, improve quality of life, and limit the socioeconomic burden of cirrhosis regardless of the etiologic factors involved.

FOOTNOTES

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Country/Territory of origin: United States

ORCID number: Seohyuk Lee 0000-0003-3023-1304; Saad Saffo 0000-0001-5375-3100.

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REVIEW

Emerging novel targets for nonalcoholic fatty liver disease treatment: Evidence from recent basic studies

Guang-Yan Wang, Xiao-Yan Zhang, Chun-Jiong Wang, You-Fei Guan

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Guang-Yan Wang, Chun-Jiong Wang, Department of Physiology and Pathophysiology, Tianjin Medical University, Tianjin 300070, China

Guang-Yan Wang, Chun-Jiong Wang, The Province and Ministry Co-sponsored Collaborative Innovation Center for Medical Epigenetics, Tianjin 300070, China

Guang-Yan Wang, Chun-Jiong Wang, NHC Key Laboratory of Hormones and Development, Chu Hsien-I Memorial Hospital and Tianjin Institute of Endocrinology, Tianjin 300070, China

Xiao-Yan Zhang, Health Science Center, East China Normal University, Shanghai 200241, China

You-Fei Guan, Advanced Institute for Medical Sciences, Dalian Medical University, Dalian 116044, Liaoning Province, China

You-Fei Guan, Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Dalian Medical University, Dalian 116044, Liaoning Province, China

You-Fei Guan, Dalian Key Laboratory for Nuclear Receptors in Major Metabolic Diseases, Dalian 116044, Liaoning Province, China

Corresponding author: You-Fei Guan, MD, Professor, Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Dalian Medical University, No. 9 West Section of Lushun South Road, Lushunkou District, Dalian 116044, Liaoning Province, China. guanyf@dmu.edu.cn

Abstract

Nonalcoholic fatty liver disease (NAFLD), a leading chronic disease worldwide, affects approximately a quarter of the global population. Nonalcoholic steatohepatitis (NASH) is an advanced form of NAFLD and is more likely to progress to liver fibrosis than simple steatosis. NASH is also identified as the most rapidly growing cause of hepatocellular carcinoma. Although in the past decade, several phase II/III clinical trials have shown promising results in the use of novel drugs targeting lipid synthase, farnesoid X receptor signaling, peroxisome proliferator-activated receptor signaling, hepatocellular injury, and inflammatory signaling, proven pharmaceutical agents to treat NASH are still lacking. Thus, continuous exploration of the mechanism underlying the pathogenesis of NAFLD and the identification of novel therapeutic targets remain urgent tasks in the field. In the current review, we summarize studies reported in recent years that not only

provide new insights into the mechanisms of NAFLD development but also explore the possibility of treating NAFLD by targeting newly identified signaling pathways. We also discuss evidence focusing on the intrahepatic targets involved in the pathogenesis of NAFLD as well as extrahepatic targets affecting liver metabolism and function.

Key Words: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Pharmaceutical strategies; Liver microenvironment; Gut-liver axis; Adipose tissue-liver axis

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Core Tip: Because of the urgent need to develop therapeutic approaches to treat nonalcoholic fatty liver disease (NAFLD), a large body of basic research has focused on the mechanisms of NAFLD to explore the possibility of new approaches to treat the disease. The current review summarizes studies reported in recent years that not only provide new insights into the mechanisms of NAFLD development but also explore the possibility of treating NAFLD by targeting newly identified signaling pathways. Evidence focusing on the intrahepatic targets involved in the pathogenesis of NAFLD as well as extrahepatic targets affecting liver metabolism and function are discussed.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) has become a leading chronic disease worldwide, affecting approximately a quarter of the global population. Nonalcoholic steatohepatitis (NASH), the advanced form of NAFLD, is closely related to liver fibrosis and even cirrhosis[1]. NASH has also been identified as the most rapidly growing cause of hepatocellular carcinoma (HCC) in liver transplant candidates in the United States[2]. Multiple factors, including disturbed lipid homeostasis, insulin resistance, and inflammation, lead to metabolic stress in hepatocytes and subsequent hepatocyte injury in NAFLD. Hepatocyte injury further triggers the wound-healing response, which involves immune cells, liver sinusoidal endothelial cells (LSECs), hepatic stellate cells (HSCs) and cholangiocytes, eventually leading to liver inflammation and fibrosis[3].

Despite the increasing prevalence of NASH, the United States Food and Drug Administration (FDA) has not yet approved any pharmaceuticals to treat patients with NASH. The mainstay of the current clinical recommendation for patients with NASH is lifestyle modification, with optimization of dietary structure, control of excessive consumption of fructose and fats and increased physical exercise, which may alleviate the progression of NASH to a certain extent[4]. In addition, bariatric surgery may be indicated for some patients with NASH suffering from severe obesity[5]. Regarding the pharmacotherapy for NASH, several biological pathways critical for glycolipid and bile acid metabolism, inflammation, hepatocellular damage (oxidative stress) and liver fibrosis have been explored as drug targets. Pharmaceutical agents, including modulators of farnesoid X receptor, peroxisome proliferator-activated receptors (PPARs), fibroblast growth factor, acetyl-CoA carboxylase (ACC), and apoptosis signal-regulating kinase 1 (ASK1), have been shown to exhibit some positive effects against NAFLD/NASH with various limitations in multiple clinical trials[6]. Thus, there is still an unmet clinical need to identify and validate novel targets for the treatment of NAFLD/NASH.

Recently, numerous novel therapeutic targets for NAFLD have been explored through basic research. These studies have focused on different pathophysiologic processes in NAFLD, including metabolic stress, liver inflammation, liver fibrosis and NASH-associated HCC. Diverse liver cells have been studied, such as hepatocytes, liver immune cells, HSCs and LSECs (Figure 1). As a metabolic disorder, crosstalk between the liver and extrahepatic organs, including the gut, adipose tissue, and skeletal muscle, largely contributes to the pathogenesis of NAFLD[7,8]. Thus, targets outside the liver broaden potential approaches to ameliorate NAFLD (Figure 1). The drugs in phase II/III clinical trials were extensively discussed in a recently published review[6]. In the present review, we summarize recent findings that not only provide new insights into the mechanisms of NAFLD development but also explore the possibility of treating NAFLD by targeting novel signaling pathways.

APPROACHES TARGETING METABOLIC STRESS TO PREVENT NAFLD PROGRESSION

Lipid accumulation in hepatocytes is a major feature of NAFLD. Disturbance of lipid and glucose metabolism causes metabolic stress in the liver. Therapeutic candidates directly regulating fatty acid metabolism that are currently in clinical trials include ACC, fatty acid synthase and stearoyl-CoA desaturase-1 inhibitors[6,9]. In addition to the targets acting on these lipid metabolic enzymes, in-depth basic research has uncovered multiple mechanisms underlying the metabolic disturbance during NAFLD, including but not limited to lipid, glucose, lactate and fructose metabolism, and has provided novel targets for NAFLD treatment.

Sterol regulatory element-binding proteins (SREBPs), including SREBP1a, SREBP1c and SREBP2, are key regulators mediating free fatty acid, triglyceride (TG) and cholesterol synthesis. 25-Hydroxanoanosterol (25-HL) is a newly identified SREBP inhibitor that induces the SCAP-INSIG interaction, which retains SREBPs in the endoplasmic reticulum (ER). 25-HL reduces hepatic TG and cholesterol levels and improves liver inflammation and fibrosis in western diet-fed mice[10]. Orosomucoid (ORM) 2, a secreted protein, has been shown to inhibit SREBP1c by activating AMP-activated protein kinase (AMPK). Recombinant ORM2 protein or stabilized ORM2–FC fusion protein inhibits lipogenesis and improves steatohepatitis[11].

Citrate regulates lipid metabolism as the substrate for lipogenesis. In the cytoplasm, citrate is catabolized by ATP-citrate lyase (ACLY) to generate acetyl-CoA and oxaloacetate. Acetyl-CoA is then converted to malonyl-CoA by ACC to fuel lipogenesis. The mitochondrial citrate carrier Slc25al plays an important role in regulating cytoplasmic and mitochondrial citrate pools. Tan and colleagues revealed that an inhibitor of Slc25a1, CTPI-2, inhibits the lipogenic pathway and prevents hepatic lipid accumulation in high-fat diet (HFD)-fed mice. Additionally, CTPI-2 also showed beneficial effects on liver inflammation[12]. ACLY, the enzyme mediating the production of acetyl-CoA from citrate, has been identified to be upregulated in the transition from simple steatosis to NASH[13]. Hepatocyte-specific deletion of ACLY reduces liver fatty acid and sterol synthesis, increases fatty acid oxidation, and attenuates glucose intolerance, liver steatosis, and ballooning[14]. Consistent with the observations following genetic inhibition, bempedoic acid, a pharmacological inhibitor of ACLY, also reduces fibrosis [14]. During the de novo lipogenesis process, nicotinamide adenine dinucleotide phosphate (NADPH), as an electron donor, aids in the reduction of acetyl-CoA. Thus, fine-tuning the NADPH pool can regulate lipogenesis. One-carbon units come largely from serine catabolism by the enzyme serine hydroxymethyltransferase (SHMT)[15]. Zhang e*t al*[16] demonstrated that SHMT1-driven serine catabolism is a substantial NADPH source in the liver. They further found that inhibition of serine catabolism by Shmt1 gene knockout or pharmacological inhibition of SHMT1/2 enzymes significantly decreased hepatic lipogenesis[16]. Moreover, in sucrose-induced fatty liver, lipogenesis also requires NADPH from serine catabolism, which can be inhibited by the SHMT1/2 inhibitor SHIN2 IV. Therefore, SHMT1/2 inhibition has the potential to treat NAFLD by decreasing de novo lipogenesis.

As an energy source and an important glycolysis product, lactate is increased in the plasma and liver of NAFLD individuals. A recent study showed that the acetylation of lactate dehydrogenase B (LDHB) is markedly increased in the livers of mice with NAFLD, which leads to decreased LDHB activity and increased hepatic lactate levels. The authors further found that P300/CBP-associated factor (PCAF)-mediated LDHB acetylation at K82 exacerbates lipid accumulation and inflammatory responses in HFD-fed mice. Consistently, embelin, an inhibitor of PCAF, significantly improves hepatic steatosis and inflammation in NASH mice[17]. In addition, increasing evidence has demonstrated that overconsumption of fructose is associated with NAFLD. Fructose is metabolized to fructose-1-phosphate by ketohexokinase. PF-06835919 is a ketohexokinase inhibitor that showed protective effects against fructose-induced liver steatosis. Moreover, the safety of PF-06835919 has been verified in a phase I clinical trial[18].

Mitochondrial and ER functions are crucial in lipid and glucose metabolism, and mitochondrial dysfunction and ER stress are closely related to NAFLD[19]. Methylation-controlled J (MCJ) protein is located at the inner mitochondrial membrane and restrains mitochondrial respiration. Its expression level is found to be increased in NAFLD patients. MCJ deficiency reduces hepatic steatosis and improves liver fibrosis in methionine- and choline-deficient (MCD) diet-fed mice. In a recent study, lipid-nanoparticle-encapsulated (LNP) siRNA was employed to target MCJ *in vivo*. LNP-siMCJ increased the β-oxidation of fatty acids and ameliorated lipid accumulation and liver fibrosis in MCD diet- or high-fat/high-fructose diet-induced NASH mice. To further specifically target MCJ in hepatocytes, N-acetylgalactosamine (GalNAc)-modified siMCJ was used to treat NASH mice. Consistent with LNP-siMCJ, GalNAc-siMCJ also reduced liver steatosis and fibrosis[20]. In addition, low-dose sorafenib, the first small-molecule multi-kinase inhibitor, was reported to induce mitochondrial uncoupling and subsequently suppress free fatty acid-induced lipid accumulation and inflammation by activating AMPK in hepatocytes. Low-dose sorafenib protects high-fat/high-cholesterol diet-fed mice against liver steatosis, inflammation and fibrosis and prevents the onset of NASH-associated HCC in mice. Notably, the beneficial effects of low-dose sorafenib were also confirmed in NASH monkeys[21]. Moreover, cyclophilin D is located in the mitochondrial matrix and mediates mitochondrial permeability transition pore opening. Cyclophilin D is upregulated in HFD-fed mice, which induces mitochondrial stress and hepatic steatosis. Cyclosporine A, an inhibitor of

cyclophilin D, decreased liver TG levels in HFD-fed mice by decreasing SREBP1c expression[22]. ER stress also plays important roles in NAFLD. The expression of forkhead box A3 (FOXA3) is induced by ER stress, and FOXA3 mediates ER stress-induced liver steatosis. As expected, targeting FOXA3 *via* a siRNA-based approach attenuates liver steatosis in HFD-fed mice[23].

It has been previously reported that the expression of DEAD-box protein 5 (DDX5), an ATP-dependent RNA helicase, is reduced in the livers of patients and mouse models with NASH, as well as in rodent models with NASH-HCC[24]. Hepatic DDX5 improves lipid metabolism by inhibiting mammalian target of rapamycin complex 1 (mTORC1) activation *via* recruitment of the tuberous sclerosis complex (TSC)1/2 complex to mTOR[24]. Through screening a natural compound library, hyperforcinol K was found to upregulate DDX5 expression by blocking tripartite motif protein 5-mediated ubiquitinated degradation of DDX5. In an animal study, hyperforcinol K was found to be effective in attenuating lipid accumulation in the livers of NASH mice[24].

Sterile 20-type kinase serine/threonine kinase 25 (STK25) has been demonstrated to play important roles in liver lipid partitioning[25,26] and systemic glucose and insulin homeostasis[27]. The level of STK25 protein is positively correlated with the development of NASH in patients[28]. Moreover, STK25 deficiency can protect against liver steatosis, inflammation, and fibrosis in mouse models of NASH-HCC[29]. STK25 antisense oligonucleotides (ASOs) significantly improved the NASH phenotype, and this preclinical validation of the effective metabolic efficacy of pharmacological inhibition of STK25 merits recognition[28].

Many nuclear receptors play critical roles in metabolic regulation. In current phase II and III trials, PPARs, farnesoid X receptor, and thyroid hormone receptor-β are promising candidate targets for NASH treatment[6]. Based on recent basic studies, modulation of retinoic acid receptor-related orphan receptor alpha (RORa) provides a novel target to treat NASH. RS-2982, a newly identified agonist of RORa, exhibited beneficial effects on reducing body weight and hepatic steatosis in HFD-fed mice. Furthermore, RS-2982 decreased alanine aminotransferase (ALT) and aspartate aminotransferase levels and reduced liver inflammation and fibrosis in mice fed an atherogenic diet. Mechanistically, an increase in miR-122 expression may account for these beneficial effects of RS-2982 on obesity and NAFLD/NASH[30].

Arachidonic acid (ARA) is an ω-6 long-chain polyunsaturated fatty acid (PUFA) that can be catalyzed into a series of bioactive eicosanoids *via* cycloxygenases (COX-1 and COX-2) and microsomal prostaglandin E synthases (mPGES-1 and mPGES-2). It has been recently reported that mPGES-2 deficiency exhibits significant protective effects against diet-induced NASH-associated phenotypes, including hepatic steatosis, inflammation and fibrosis. However, the beneficial effect of mPGES-2 deficiency against NAFLD is dependent on decreased cytochrome P450 4A14 (CYP4A14) and increased acyl-CoA thioesterase 4 levels but not PGE2[31]. Mechanistically, mPGES-2 binds with heme, which is released after mPGES2 inhibition and in turn activates the heme receptor nuclear receptor subfamily 1 group D member 1 (NR1D1) to upregulate CYP4A14 and acyl-CoA thioesterase 4 expression[31]. CYP4A14 catalyzes omega-hydroxylation of medium-chain fatty acids and ARA in mice. Its expression was previously found to be significantly increased in the livers of patients and mice with NAFLD. Loss of CYP4A14 function also markedly attenuated liver damage, inflammation, and fibrosis in MCD diet-induced NASH[32]. Moreover, both the mPGES-2 inhibitor SZ0232 and the CYP4A inhibitor TS-011 are capable of ameliorating the NASH phenotype in MCD diet-fed mice[31-33]. Taken together, these findings demonstrate that ARA metabolic enzymes play critical roles in liver lipid homeostasis and represent attractive targets for developing therapeutic drugs for NAFLD/NASH.

Emerging evidence suggests that novel therapeutic targets can be developed based on the discovery of genetic variants related to liver lipid metabolism, such as *PNPLA3*, *TM6SF2*, *MBOAT7* and *HSD17B13* [34-37]. It has been reported that phosphorylation of HSD17B13 at serine 33 by PKA promotes lipolysis. Reproterol, a β2 agonist used for treating asthma, protects against the NASH phenotype *via* PKA-mediated Ser33 phosphorylation of 17β-HSD13[38]. Most recently, a variant in the *pleckstrin and Sec7 domain-containing 3* (*PSD3*) gene, rs71519934, was reported to reduce susceptibility to fatty liver disease, consistent with the finding that the expression level of *PSD3* is increased in patients with NAFLD and that knockdown of *PSD3* by siRNA decreases TG synthesis in hepatocytes. Furthermore, specific downregulation of *PSD3* in hepatocytes by GalNAc-conjugated ASOs resulted in decreased hepatic lipid content and plasma ALT levels and improved liver fibrosis in NASH mice[39], suggesting that *PSD3* may be a potential therapeutic target for the treatment of NAFLD/NASH.

STRATEGIES PROTECTING HEPATOCYTES FROM INJURY DURING NASH DEVELOPMENT

Lipid accumulation in hepatocytes can cause cytotoxicity (lipotoxicity) in NAFLD[40,41]. Hepatocyte injury is a key event during the development of NASH, and ballooned hepatocytes are an essential feature to diagnose NASH. In phase II/III clinical trials, pancaspase inhibitors and ASK1 inhibitors are currently under evaluation as therapeutic agents directly targeting apoptosis[6]. Studies focused on developing targets to prevent hepatocyte injury have also shown therapeutic effects in NASH.

Mitochondrial stress is a major factor driving hepatocyte injury and promotes the progression from simple steatosis to NASH. Mitochondrial matrix caseinolytic protease P (ClpP) is a protease component of the caseinolytic protease complex that maintains protein homeostasis in mitochondria. Its expression level is frequently downregulated in NASH, which induces mitochondrial stress and inflammation in hepatocytes. The ClpP activator A54556A is capable of greatly ameliorating the NASH phenotype in high-fat/high-fructose diet-fed mice[42]. SH3 homology-associated BTK binding protein (SAB) was originally identified as a phosphorylated c-Jun N-terminal kinase (p-JNK) docking protein and a substrate of JNK in the mitochondrial outer membrane. The interaction of JNK with SAB leads to increased mitochondrial reactive oxygen species (ROS) production and promotes liver injury. In established NASH, hepatocyte-targeted GalNAc-Sab-ASO treatment can reverse steatohepatitis and fibrosis by abrogating the adverse effects of the JNK-SAB-ROS activation loop[43]. Sirtuin activation plays an important role in maintaining mitochondrial homeostasis and shows protective effects on NAFLD[44]. Increased NAD^+ levels can activate sirtuin. The enzyme α-amino-β-carboxymuconate-ε-semialdehyde decarboxylase controls NAD^+ levels, and its inhibitor TES-991 boosts de novo NAD^+ synthesis and protects mice against liver injury and steatosis by improving mitochondrial function[45]. In addition, the serine/threonine protein phosphatase 2A inhibitors LB100[46], allyl isothiocyanate[47], celastrol[48] and the heme oxygenase inducer cobalt protoporphyrin[49] have all been found to significantly ameliorate NASH *via* the Sirt1 pathway. Nuclear factor erythroid 2–related factor 2 (Nrf2) is a well-known important transcription factor involved in the expression of antioxidant genes during oxidative stress. Targeted deletion of Nrf2 results in enhanced susceptibility to a variety of oxidative stress-induced liver injuries, including NASH. In contrast, pharmacological or genetic activation of hepatic Nrf2 leads to hepatic protection against oxidative damage. Studies have shown beneficial effects of hepatocyte Nrf2 activation in NASH[50]. Pharmacological activation of Nrf2 by the acetylenic tricyclic bis (cyano enone) compounds TBE-31[51], NK-252[52], and dimethyl fumarate[53] suppresses NASH. These findings demonstrate that strategies that increase Nrf2 expression and activity may be attractive strategies to limit the development of NAFLD/NASH by attenuating hepatocyte lipotoxicity and injury.

As mentioned above, apoptosis plays an important role in hepatocyte injury or loss during the development of NAFLD/NASH. BCL-2 belongs to the BCL-2 family, which is antiapoptotic. Through screening a small molecule library, acridone derivative A22 was found to increase BCL-2 expression by stabilizing the BCL-2 promoter i-motif. As expected, A22 can attenuate HFD-induced hepatocyte injury, hepatic steatosis and liver fibrosis[54]. In addition, necroptosis, another form of programmed cell death, was found to be increased in NASH mouse models and the livers of NAFLD patients. Receptor-interacting protein 3 (RIP3) and receptor-interacting protein kinase (RIPK) 1 are key mediators of necroptosis. Liver RIP3 deficiency can attenuate MCD diet-induced liver injury, steatosis, inflammation and fibrosis[55]. Similarly, the RIPK1 inhibitor RIPA-56 can reduce liver inflammation and fibrosis in HFD-fed mice by abrogating necroptosis. In addition, RIPA-56 is able to ameliorate hepatic steatosis by suppressing mixed lineage kinase domain-like protein levels[56].

Iron overload leads to hepatic oxidative stress and hepatocellular ballooning injury and plays a multifactorial role in the pathogenesis of NASH[57]. Approximately one-third of patients with NAFLD show interrupted iron homeostasis[58]. Ferroptosis is a nonapoptotic programmed cell death process characterized by iron-dependent and lipid peroxidation-associated cell death[59]. The ferroptosis inhibitors Trolox and deferiprone can protect hepatocytes from cell death and suppress the subsequent initiation of inflammation in fatty liver[60].

In contrast to hepatocytes, agents that induce HSC apoptosis show beneficial effects against NASH-related liver fibrosis[61]. Thus, when using pharmacological agents to prevent hepatocyte injury, strategies more accurately targeting hepatocytes may strengthen the therapeutic effects of these agents against NASH with limited side effects.

APPROACHES TARGETING THE INFLAMMATORY PATHWAY TO TREAT NAFLD/NASH

Inflammatory responses mediated by various immune cells and hepatocytes promote the onset of NASH and liver fibrosis. Infiltration of neutrophils and macrophages is the main pathological feature of NASH. Neutrophil depletion attenuates diet-induced NASH[62]. CXC chemokine receptor 2 (CXCR2) signaling is considered to play a pivotal role in the entry of neutrophils into peripheral tissues. Leslie and colleagues demonstrated that human and mouse livers with NASH-HCC have more $CXCR2^+$ neutrophils[63]. The expression of CXCR2 in neutrophils is induced in NASH in an autocrine manner involving the upregulation of neutrophil-derived lipocalin 2[64]. AZD5069 is a small-molecule inhibitor of CXCR2. AZD5069 can significantly improve liver pathology in NAFLD, with reduced lipid content and hepatic neutrophil accumulation and improved insulin sensitivity[65]. In addition, AZD5069-mediated CXCR2 inhibition induces reprogramming of the tumor immune microenvironment, which promotes immune checkpoint inhibition in NASH-HCC[63], suggesting that blocking infiltrating neutrophil CXCR2 can restore sensitivity to immunotherapy in the NASH liver[66].

It is well known that the expression of macrophage scavenger receptor 1 (MSR1) mediates lipid uptake in macrophages and subsequently induces inflammatory activation *via* the JNK pathway. MSR1 was found to be correlated with liver inflammation in NAFLD patients[67]. Notably, therapeutic inhibition of MSR1 with an anti-MSR1 antibody improved hepatic steatosis in a mouse model of NASH. The number of F4/80-positive cells and the expression level of tumor necrosis factor alpha (TNF-α) were also found to be reduced by the anti-MSR1 antibody therapy[67].

It has long been recognized that the NOD-like receptor protein 3 (NLRP3) inflammasome is activated in NASH livers, which leads to increased inflammation and programmed cell death. MCC950, an NLRP3 inhibitor, can improve NAFLD pathology and fibrosis in obese diabetic mice[68]. X-box binding protein-1 (XBP1) is a key factor regulating the unfolded protein response. Macrophage XBP1 is upregulated in NASH livers, which activates macrophage NLRP3 signaling and promotes hepatocyte steatosis and HSC activation. As expected, the XBP1 inhibitor toyocamycin exhibits protective effects against NASH[69].

Nuclear factor-kappaB (NF-κB) is an essential transcription factor that mediates the inflammatory response and proinflammatory cytokine production in NASH[70]. miR-378 markedly facilitates the NF-κB-TNF-α axis in NASH development by directly targeting the *Prkag2* gene, which encodes AMPK-γ2. Similarly, downregulation of miR-378 by ASO is also capable of alleviating NASH development[71].

In addition to neutrophils and macrophages, other immune cells, including dendritic cells (DCs) and lymphocytes, play pivotal roles in NASH[8]. DC recruitment to the liver promotes the inflammatory response in NAFLD progression. Reportedly, delivery of liposomal curcumin or calcitriol to lipid-rich inflammatory DCs shifted their inflammatory profile toward a regulatory phenotype and improved hepatic steatosis, inflammation and fibrosis in a NASH mouse model[72]. Inflammatory CX_3CR1^+ monocyte-derived inflammatory DCs (moDCs) are found to contribute to sustained inflammation and liver injury during NASH. Treating MCD-fed mice with the hydrogen sulfide donor *i.e.*, sodium hydrosulphide, prevented the accumulation of CX_3CR1^+ moDCs and ameliorated parenchymal injury [73]. Moreover, Toll-like receptor 7 (TLR7) signaling can induce proinflammatory cytokine production in Kupffer cells and DCs, subsequently suppressing regulatory T cells (Tregs) and leading to steatohepatitis. Notably, treatment with IRS-661, a TLR7 antagonist, could ameliorate NASH development[74]. It has been reported that α4β7-mediated homing of CD4 T cells to the intestine and liver facilitates NASH development, and α4β7 blockade by neutralizing monoclonal antibody can attenuate hepatic inflammation and fibrosis and improve metabolic dysfunction associated with NASH[75]. In addition, a high level of Tregs reportedly promotes the initiation and progression of cancer in NASH livers. In a NASH-HCC model, anti-CD25 antibodies, capable of alternatively depleting Tregs, decreases the tumor burden and increased survival time[76]. The interaction of B2 Lymphocytes with T cells contributes to NASH progression, B-cell activating factor-neutralizing monoclonal antibody Sandy-2 prevented hepatic B2 cell response and ameliorated the evolution of NASH in mice[77].

Bioactive lipids are important nodes in lipid metabolism and tissue homeostasis networks[78]. An imbalance between protective and deteriorative bioactive lipids contributes to NASH progression[79]. Pharmacological targeting of the synthesizing enzymes or receptors of these bioactive lipids has shown beneficial effects in NASH. Leukotriene B4 (LTB4), a proinflammatory metabolite derived from the ω-6 PUFA ARA, is significantly increased in patients with NAFLD[80]. Both *in vivo* and *in vitro* experiments have shown that LTB4 can promote hepatocyte lipogenesis, which is dependent on the RNase activity of IRE1α through leukotriene B4 receptor 1 (Ltb4r1). Furthermore, LTB4/Ltb4r1 stimulation increases intracellular cAMP and then promotes IRE1α Ser724 phosphorylation by PKA[80]. The Ltb4r1 inhibitor CP-105696 significantly alleviated ER stress and dyslipidemia in NAFLD mice[80]. Unlike ω-6 PUFAs, ω-3 PUFAs and their metabolites show anti-inflammatory effects. Fat-1 mice, a transgenic animal model in which tissues are endogenously enriched with ω-3 PUFAs[81], are protected from diet-induced metabolic dysfunction and fibrosis[82]. However, they are vulnerable to lipid peroxidation, which limits their clinical applications[83]. Fraser *et al*[84] developed a structurally modified ω-3 fatty acid, icosabutate, which was designed to resist oxidation and incorporation into hepatocytes and possesses the potential to activate free fatty acid receptor 4. They found that icosabutate, but not the ω-3 PUFA eicosapentaenoic acid, can ameliorate liver inflammation and fibrosis in NASH rats. Moreover, icosabutate treatment decreases liver injury in patients at high risk of NASH and cardiovascular disease [84].

APPROACHES TARGETING HSC ACTIVATION TO PREVENT NASH-RELATED LIVER FIBROSIS

In clinical trials for NASH drug development, improvement of liver fibrosis has been frequently employed as a primary or secondary endpoint. Given the central role of HSCs in liver fibrosis, targeting HSC activation has long been proposed as a therapeutic strategy to prevent NASH-related fibrosis progression.

The Notch, Hedgehog, Hippo and WNT/β-catenin signaling pathways in hepatocytes play important roles in NASH-related liver fibrosis by modulating the hepatic microenvironment. The production of osteopontin (OPN), as a paracrine factor, from hepatocytes or cholangiocytes increases in NASH, which leads to the activation of HSCs[85-87]. Increased Notch signaling activity has been found to be responsible for the induction of OPN[85]. Thus, several studies have targeted Notch signaling to treat NASH, including studies on Nicastrin ASO and nanoparticle-mediated delivery systems targeting Notch antagonism[85,88]. In addition, nuclear factor of activated T-cell 4 (NFATc4) was also reported to induce OPN expression by negatively regulating the transcriptional activity of PPARα. As expected, inhibition of NFATc4 decreased lipid content and improved inflammation and fibrosis in NASH mice [89]. Hepatocyte transcriptional co-activator with PDZ-binding motif (TAZ) was found to promote NASH-related liver fibrosis by increasing Indian hedgehog, which activates HSCs[90]. A recent study reported that stabilized GalNAc-siRNAs targeting hepatocyte TAZ can significantly ameliorate liver inflammation and fibrosis in mice with established NASH[91]. Furthermore, WNT1-inducible signaling pathway protein 1 (WISP1), a member of the cellular communication network family, has recently been identified as an extracellular activator of the myocardin-related transcription factor-cytoskeleton pathway in HSCs. Activation of the WISP1-MRIF signaling pathway induces HSC migration and promotes liver fibrosis progression. Notably, an anti-WISP1 antibody significantly ameliorated liver fibrosis in NASH mice[92].

Lnterleukin-11 (IL-11) has been demonstrated to activate HSCs. Deletion of IL-11 receptor subunit alpha (IL-11RA) protects mice from NASH diet-induced hepatocyte death, liver inflammation and fibrosis. Widjaja *et al*[93] developed a neutralizing anti-IL-11 antibody and neutralizing anti-IL-11RA antibody and found that both of them greatly decreased ALT levels and significantly improved hepatic steatosis and liver fibrosis in mouse models of NASH[93].

Activation of the JAK/signal transducer and activator of transcription (STAT) pathway in HSCs promotes liver fibrosis. Ruxolitinib, an effective small-molecule JAK1/2 selective inhibitor, has been approved by the FDA for myelofibrosis treatment. Recently, it has been reported that ruxolitinib can block HSC activation and attenuate liver fibrosis progression[94]. In addition, the JAK2 inhibitor pacritinib affords protection against NAFLD-related liver fibrosis by inhibiting HSC activation[95]. Rilpivirine, a nonnucleoside reverse transcriptase inhibitor, is widely used to treat HIV infection. Rilpivirine can also ameliorate liver fibrosis, possibly through selective STAT1-dependent induction of apoptosis in HSCs, and suppress HFD- and CCl4-induced liver fibrosis. In addition, rilpivirine enhances STAT3-dependent proliferation in hepatocytes as an effect secondary to its pro-apoptotic effect in HSCs [96].

Protease-activated receptor-2 (PAR2) is an emerging new target for NASH that regulates liver injury, inflammation and fibrosis and plays a critical role in regulating hepatic cholesterol and glucolipid metabolism[97-99]. Pharmacological inhibition of PAR2 with pepducin PZ-235, a full antagonist of PAR2, not only protects against the activation of HSCs and fibrosis but also promotes hepatocellular viability by inhibiting mitochondrial ROS production induced by PAR2 stimulation[97].

APPROACHES TARGETING LSECS AND CHOLANGIOCYTES TO TREAT NAFLD

LSECs are highly specialized endothelial cells with fenestrae and lack a basement membrane. LSECs interact with hepatocytes, liver immune cells and HSCs and play important roles in regulating liver function. LSEC dysfunction, including LSEC capillarization, disturbed nitric oxide release from LSECs, and increased expression of adhesion molecules, all contribute to NAFLD pathogenesis[100]. Vascular cell adhesion molecule 1 (VCAM-1) is an adhesion molecule upregulated in LSECs in NASH mouse livers. Anti-VCAM1 antibody attenuates hepatic inflammation in NASH mice[101]. Endothelial nitric oxide synthase (eNOS) expression in LSECs is decreased in NASH mice, which is likely the result of Notch activation. Pharmacological inhibition of Notch signaling using DAPT and LY3039478, as well as the eNOS activator YC-1, improved the NASH phenotype in MCD diet-fed mice[102]. Although the role of LESCs in NAFLD has been increasingly emphasized, the underlying mechanisms and therapeutic approaches targeting LESCs need more exploration.

Cholangiocytes also play an important role in NASH development. In addition to hepatocytes, OPN is strongly expressed in cholangiocytes in NASH livers. Thus, it is highly possible that cholangiocyte-derived OPN can promote liver fibrosis by activating HSCs[87]. Cardiotrophin-like cytokine factor 1 is another cholangiocyte-derived paracrine factor that exerts beneficial effects in NASH. Downregulation of a subunit of its receptor complex leukemia inhibitory factor receptor contributes to the progression of NASH[103]. Compared with other nonparenchymal cells, there are fewer studies addressing the effects of cholangiocytes on NASH, and a related therapeutic approach is still lacking.

POTENTIAL TARGETS OUTSIDE THE LIVER

It is well-established that multiple organs form a network to regulate whole-body energy metabolism.

Extrahepatic organs influence liver function in an endocrine manner by releasing hormones or inflammatory factors or by regulating systemic metabolic homeostasis such as energy expenditure and insulin sensitivity. Thus, targeting these extrahepatic organs provides additional options for treating NAFLD/NASH.

Targets affect systemic energy metabolism

Diets with reduced contents of sugars, refined carbohydrates and saturated fat are recommended to treat NAFLD[104]. Moreover, exercise is considered as an effective strategy for preventing and treating NAFLD *via* metabolic pathways and interorgan crosstalk[105]. Glucagon-like peptide (GLP)-1 receptor agonists, which enhance insulin secretion and reduce food intake, show beneficial effects against NASH in clinical trials[6]. In addition, fibroblast growth factor 21 analogs improved systemic energy metabolism and demonstrated potential in NASH therapy[6]. In a recent basic study, a novel Zn supplement, ulvan oligosaccharide (UO)-Zn, can activate AMPK, leading to improved metabolic pathways in HFD-fed mice[106]. The increased hepatic expression of branched chain ketoacid dehydrogenase kinase (BDK) partly induced high levels of branched-chain amino acids (BCAA) in metabolic diseases[107-109]. Moreover, 3,6-dichlorobrenzo(b)thiophene-2-carboxylic acid, a small-molecule allosteric inhibitor of BDK, lowered levels of circulating BCAAs, reduced hepatic steatosis, and improved glucose tolerance in rodent models[107,108].

The gut-liver axis

The crosstalk between the gut and liver is regarded as an important pathway in regulating hepatic functions. Intestinal inflammation and dysfunction of intestinal lipid and bile acid absorption, epithelial cell permeability and the microbiome affect liver function and contribute to NAFLD progression. The relationship between gut microbiota and potential therapeutic targets has been recently discussed in depth elsewhere[110,111]; thus, we only focus on other aspects of the gut-liver axis.

Serum ceramide levels are positively related to NAFLD, and intestine-derived ceramide has been demonstrated to promote NAFLD[112,113]. Intestinal hypoxia-inducible factor 2α (HIF-2α) and myelocytomatosis oncogene (MYC) affect NAFLD by regulating ceramide metabolism. Intestinal HIF-2α , but not HIF-1α, is activated in obese patients and mice. Intestine-specific HIF-2α knockout improves hepatic steatosis by decreasing intestine-derived ceramide levels. Mechanistically, HIF-2α transcriptionally upregulates the expression of Neu3, a key enzyme in the ceramide salvage pathway. Targeting HIF-2α with its inhibitor PT2385 significantly attenuated hepatic lipid accumulation and decreased plasma ALT levels in HFD-fed mice[112]. Moreover, oral administration of PT2385 contributes to reduced body weight and improved insulin sensitivity[112]. Intestinal MYC expression is positively related to body mass index and serum ALT levels in humans. Genetic ablation of MYC in the intestine suppressed obesity and hepatic steatosis and led to decreased ceramide levels in HFD-fed mice. Ceramide synthase 4 has been further demonstrated to be a target gene of MYC. In line with this finding, pharmacological inhibition of MYC with 10058-F4 was found to ameliorate the metabolic disorders and liver fibrosis induced by HFD feeding[114]. In addition, increased intestinal ceramide also meditates the acceleration of NASH induced by nicotine[115].

Gut-derived 5-hydroxytryptamine (5-HT) has been reported to promote lipogenesis in the liver. Moreover, liver-specific knockout of the 5-HT receptor 2α (*Htr2α*) gene significantly lowered liver lipid content in HFD-fed mice and decreased the expression of genes involved in lipogenesis. To evaluate the potential of targeting the gut-5-HT-liver Htr2α pathway as a therapeutic strategy for NAFLD, the 5-HT receptor HTR2A antagonist sarpogrelate was used to treat HFD-fed mice. The results showed that sarpogrelate greatly ameliorates hepatic steatosis in NAFLD mice[116].

Yan *et al*[117] recently clarified that gut PPARα activation promotes NASH development by inducing fatty acid binding protein 1 expression, thereby aggravating fatty acid absorption in the small intestine [117]. GW6471, a PPARα-specific antagonist, was found to accumulate in the small intestine at much higher levels than in the liver. Intestine-specific PPARα deficiency and gut PPARα antagonism by GW6471 both improve NASH phenotypes[117]. The pleotropic roles of PPARα in different tissues in modulating NASH are worth discussing. Hepatocyte PPARα is crucial for whole-body fatty acid homeostasis and is protective against NAFLD[118]. Therapeutic strategies to prevent or treat NAFLD by activating hepatocyte PPARα and/or inhibiting gut PPARα may help guide drug delivery. Monoacylglycerol acyltransferase 2 (MGAT2) mediates the conversion of monoacylglycerol to diacylglycerol and is critical for dietary fat absorption. In the liver, MGAT2 regulates TG synthesis. BMS-963272, a potent MGAT2 inhibitor, improves the NAFLD activity score and liver fibrosis in NAFLD/NASH mice[119]. Of note, its safety has been confirmed in phase 1 clinical trials[119].

In addition, a long-acting dual agonist of GLP-1 and GLP-2 receptors, GLP1/2-Fc, has been found to greatly alter the microbiome composition and exhibits positive effects on gastrointestinal volume and the intestinal barrier. More importantly, GLP1/2-Fc can significantly ameliorate NASH phenotypes, including hepatic fat accumulation, inflammation, fibrosis and insulin tolerance[120], and its effects are superior to those of liraglutide, which has minimal efficacy on inflammation or fibrosis[121].

The adipose tissue-liver axis

Recently, several novel adipokines have been identified and verified to play important roles in NASH development and progression by mediating the crosstalk between adipose tissue and the liver. Isthmin-1 (ISM1) was identified as an adipokine that can activate phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-protein kinase B (Akt) signaling independent of insulin. *ISM1* gene expression is positively correlated with body mass index, and its deficiency leads to glucose intolerance *via* inhibition of PI3K-Akt signaling. In addition, ISM1 suppressed de novo lipogenesis in the liver. Recombinant ISM1 protein exhibited beneficial effects on both glucose tolerance and hepatic steatosis in NAFLD mice[122]. Recently, Liu *et al*[123] demonstrated that secreted protein acidic and rich in cysteine-like protein 1 (Sparcl1), which is a white adipose tissue-secreted protein, is correlated with hepatic pathological features in NASH patients. The authors further found that sparcl1 can induce liver inflammation by increasing C-C motif chemokine ligand 2 expression in the liver. To further determine the role of sparcl1 in the pathogenesis of NASH, the authors developed a neutralizing antibody against sparcl1 and found that the sparcl1-neutralizing antibody markedly ameliorated liver inflammation and liver fibrosis in NASH mice[123]. Gremlin 1, a newly identified adipokine, is positively related to insulin resistance and NAFLD/NASH. Treatment with recombinant Gremlin 1 protein impairs insulin sensitivity in various cell types, including human primary adipocytes, skeletal muscle cells, and liver cells. The insulin-sensitizing effect of the neutralizing anti–Gremlin 1 antibody indicates its beneficial effects on insulin resistance and NAFLD/NASH[124]. Neuregulin 4 (NRG4) is an adipose tissue-derived endocrine factor that has been demonstrated to suppress NASH-associated HCC by restraining the tumor-prone liver immune microenvironment. A recombinant fusion protein comprising amino acids 1–55 of human NRG4 and the Fc domain of immunoglobulin G 1 was found to suppress HCC induced by a NASH diet plus oncogene overexpression[125].

SWELL1/LRRC8a, a leucine-rich repeat-containing transmembrane protein, functionally encodes an ion channel signaling complex on the adipocyte plasma membrane[126]. Adipocyte SWELL1 is dispensable for adipose development[127]. SWELL1 protein is reduced in the adipocytes of type 2 diabetic animals. The small molecule SN-401, which binds at a constriction point within the SWELL hexamer, can increase adipocyte SWELL1 protein expression and SWELL1-dependent insulin signaling. Similarly, SN-401 can normalize glucose tolerance by augmenting tissue glucose uptake, suppressing hepatic glucose production, increasing serum fibroblast growth factor 21 levels, and reducing hepatic steatosis and hepatocyte ballooning in obese type 2 diabetic mice[128]. Taken together, these findings demonstrate that targeting the adipose tissue-liver axis may be an attractive strategy to treat NAFLD/NASH.

The skeletal muscle-liver axis

The crosstalk between skeletal muscle and the liver also impacts NAFLD progression. As one of the energy metabolism organs, skeletal muscle can affect liver lipid and glucose metabolism by regulating systemic metabolic homeostasis. In addition, decreased skeletal muscle mass and altered myokine secretion are associated with exacerbation of NAFLD progression[129,130]. Myostatin signaling negatively regulates muscle mass and is positively related to liver fibrosis[131]. Irisin (encoded by fibronectin type III domain containing 5, Fndc5), a myokine induced by exercise, is also expressed in adipose tissue and the liver. Serum irisin levels are inversely associated with TG content in the livers of obese adults[132]. Recombinant Fndc5/irisin significantly improved the NASH phenotype in HFD-fed mice[133]. The administration of nicotinamide riboside can alleviate obesity and steatosis by increasing irisin levels in HFD-fed mice[133]. Collectively, therapeutic strategies to improve skeletal muscle quantity and quality or target myokine signaling may provide promising options to treat NASH, but basic studies to collect more evidence are warranted.

CONCLUSION

Because of the urgent need to develop therapeutic approaches to treat NAFLD/NASH and the complexity of the pathophysiologic process of NAFLD, a large body of basic research has focused on the mechanisms of NAFLD to explore the possibility of applying new approaches to treat the disease (Tables 1 and 2). Metabolic stress-induced hepatocyte injury is an initial factor driving NASH development. Thus, in hepatocytes, strategies for treating NASH aim to improve metabolic disturbance and protect hepatocytes from injury. Alteration of the liver microenvironment and the interaction between diverse liver cells promote liver inflammation and fibrosis. Therefore, the inflammatory response of hepatocytes and immune cells in the liver is also an important target for NASH treatment. Strategies targeting HSC activation, proliferation and migration show beneficial effects on liver fibrosis and may become a novel way to attenuate NASH outcomes (Figure 1). As an indispensable part of hepatic metabolic regulation, extrahepatic organs play an essential role in liver lipid homeostasis, and their dysfunction contributes to the development and progression of NAFLD/NASH. The gut, adipose tissue and skeletal muscle can functionally affect liver or whole-body energy metabolic homeostasis *via* various endocrine factors (Figure 1 and Table 2).

Table 1 Novel intrahepatic targets explored by recent basic study

Molecular target	Potential agent	Classification	Function	Effects on NAFLD	Ref.
SREBPs	25-HL	Inhibitor	Decrease TG and cholesterol synthesis	-Hepatic steatosis -Liver inflammation -Liver fibrosis	[10]
ORM2	Recombinant ORM2; ORM2-FC fusion protein	Recombinant protein	Inhibit lipogenesis	-Hepatic steatosis -Liver inflammation -Liver fibrosis	[11]
Slc25al	CTPI-2	Inhibitor	Regulating cytoplasmic and mitochondrial citrate pool	-Hepatic steatosis -Liver inflammation	[12]
ACLY	Bempedoic acid	Inhibitor	Inhibit ACLY activity	-Hepatic steatosis -Liver injury	[14]
SHMT1/2	SHIN2 IV	Inhibitor	Decrease serine catabolism derived NADPH	-Hepatic lipogenesis	[16]
PCAF	Embelin	Inhibitor	Inhibit acetylation of LDHB	-Hepatic steatosis -Liver inflammation	[17]
Ketohexokinase	PF-06835919	Inhibitor	Regulating fructose metabolism	-Fructose-induced liver steatosis	[18]
Mitochondrial uncoupling	Sorafenib	Small molecule	Induce mitochondrial uncoupling and activate AMPK	-Hepatic steatosis -Liver inflammation -Liver fibrosis -NASH-associated HCC	[21]
MCJ	LNP-siMCJ; GalNAc-siMCJ	Nucleic acid-based therapy	Maintain mitochondrial function	+β-oxidation -Hepatic steatosis -Liver fibrosis	[20]
Cyclophilin D	CsA	Inhibitor		-Hepatic steatosis	[22]
ClpP	A54556A	Activator		-Hepatic steatosis -Liver inflammation -Liver fibrosis	[42]
SAB	GalNAc-Sab ASO	Nucleic acid-based therapy		-Hepatic steatosis -Liver inflammation -Liver fibrosis	[43]
ACMSD	TES-991	Inhibitor		-Hepatic steatosis -Liver inflammation	[45]
FOXA3	FOXA3 siRNA	Nucleic acid-based therapy	Attenuate ER stress induced liver steatosis	-Hepatic steatosis	[23]
TRIM5-DDX5	Hyperforcinol K	N/A	Inhibit ubiquitinated degradation of DDX5	-Hepatic steatosis -Liver inflammation	[24]
STK25	STK25 ASO	Nucleic acid-based therapy	Regulating energy homeostasis	-Hepatic steatosis -Liver inflammation -Liver fibrosis	[28]
RORa	RS-2982	Agonist	Increase miR-122 level	-Hepatic steatosis -Liver inflammation	[30]

				-Liver fibrosis	
HSD17B13	Reproterol	Small molecule	Induce the Ser33 phosphorylation of 17β-HSD13 protein	-Hepatic steatosis -Liver fibrosis	[38]
PSD3	GalNAc-Psd3 ASO	Nucleic acid-based therapy	Decrease TG synthesis	-Hepatic steatosis -Liver injury -Liver fibrosis	[39]
Nrf2	TBE-31; NK-252; Dimethyl fumarate	Activator	Reduce oxidative stress	-Hepatic steatosis -Liver inflammation -Liver fibrosis	[51-53]
BCL-2	A22	Small molecule	Anti-apoptosis	-Liver injury -Liver fibrosis	[54]
RIPK1	RIPA-56	Inhibitor	Abrogating necroptosis	-Hepatic steatosis -Liver inflammation	[56]
CXCR2	AZD5069	Inhibitor	Inhibit neutrophil infiltration	-Hepatic steatosis -Liver inflammation -Liver fibrosis -NASH-associated HCC	[63,65]
MSR1	Anti-MSR1 antibody	Neutralizing antibody	Inhibit inflammatory response	-Hepatic steatosis -Liver inflammation	[67]
NLRP3	MCC950	Inhibitor	Inhibit NLRP3 activation	-Liver inflammation -Liver fibrosis	[68]
XBP1	Toyocamycin	Inhibitor		-Hepatic steatosis -Liver inflammation -Liver fibrosis	[69]
DCs	Curcumin; calcitriol	Small molecule	Shift hepatic DC inflammatory profile toward a regulatory phenotype	-Hepatic steatosis -Liver inflammation -Liver fibrosis	[72]
Hydrogen sulfide	NaHS	Hydrogen sulfide donor	Prevent the accumulation of TNF-α-producing CX_3CR1^+ moDCs	-Liver injury	[73]
TLR7	IRS-661	Antagonist	Decrease proinflammatory cytokine production in Kupffer cells and DCs	-Hepatic steatosis -Liver inflammation	[74]
Integrin	Anti-α4β7 antibody	Neutralizing antibody	Decrease $\alpha4\beta7^+$ CD4 T-cell recruitment	-Hepatic steatosis -Liver inflammation -Liver fibrosis	[75]
Tregs	Anti-CD25 antibodies	Neutralizing antibody	Deplete Tregs	-NASH-associated HCC	[76]
BAFF	Sandy-2	Neutralizing antibody	Prevent hepatic B2-cell responses	-Hepatic steatosis -Liver inflammation -Liver fibrosis	[77]
Notch	Nicastrin ASO	Nucleic acid-based therapy	Inhibit Notch signaling	-Liver fibrosis	[85]
	NP- dibenzazepine	Inhibitor with target delivery system		-Liver fibrosis	[88]
TAZ	GalNAc-TAZ siRNA	Nucleic acid-based	Inhibit TAZ in hepatocyte	-Liver inflammation	[91]

WISP1	Anti-WISP1 antibody	therapy	and inhibit HSC activation	-Liver fibrosis	[92]
		Neutralizing antibody	Inhibit HSC migration	-Liver fibrosis	
IL-11	Anti-IL-11 antibody	Neutralizing antibody	Inhibit HSC activation; inhibit hepatocyte injury	-Liver injury -Liver fibrosis	[93]
JAK1/2	Ruxolitinib	Inhibitor	Inhibit HSC activation	-Liver fibrosis	[94]
JAK2	Pacritinib	Inhibitor	Inhibit HSC activation	-Liver fibrosis	[95]
STAT1	Rilpivirine	Inhibitor	Induce HSC apoptosis and promote hepatocyte proliferation	-Liver fibrosis	[96]
PAR2	Pepducin PZ-235	Antagonist	Inhibit HSC activation	-Liver injury -Liver fibrosis	[97]
VCAM-1	Anti-VCAM-1 antibody	Neutralizing antibody	Suppress monocyte adhesion to LSEC	-Liver inflammation -Liver fibrosis	[101]
eNOS	YC-1	Activator	Increase NO production from LSEC	-Hepatic steatosis -Liver inflammation -Liver fibrosis	[102]
Ltb4r1	CP-105696	Inhibitor	Inhibit the effects of LTB4	-Hepatic steatosis -Liver inflammation -Liver fibrosis	[80]
mPGES-2	SZ0232	Inhibitor	Regulating ARA metabolism	-Hepatic steatosis	[31]
CYP4A	TS-011	Inhibitor		-Hepatic steatosis -Liver inflammation -Liver fibrosis	[32]
N/A	Icosabutate	Structurally modified ω-3 fatty acid	Resist oxidation and activate free fatty acid receptor 4	-Liver inflammation -Liver fibrosis	[84]

-: Inhibit; +: Stimulate; SREBPs: Sterol regulatory element-binding proteins; TG: Triglyceride; 25-HL: 25-Hydroxanoanosterol; HSC: Hepatic stellate cell; LSEC: Liver sinusoidal endothelial cell; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; NO: Nitric oxide; N/A: No application; ORM2: Orosomucoid 2; ACLY: ATP-citrate lyase; SHMT1/2: Serine hydroxymethyltransferase 1/2; NADPH: Nicotinamide adenine dinucleotide phosphate; PCAF: P300/CBP-associated factor; LDHB: Lactate dehydrogenase B; AMPK: AMP-activated protein kinase; HCC: Hepatocellular carcinoma; MCJ: Methylation-controlled J; LNP: Lipid-nanoparticle-encapsulated; GalNAc: N-acetylgalactosamine; ClpP: Caseinolytic protease P; SAB: SH3 homology-associated BTK binding protein; ASO: Antisense oligonucleotides; ACMSD: α-amino-β-carboxymuconate-ε-semialdehyde decarboxylase; FOXA3: Forkhead box A3; STK25: Sterile 20-type kinase serine/threonine kinase 25; RORa: Receptor-related orphan receptor alpha; PSD3: Pleckstrin and Sec7 domain-containing 3; Nrf2: Nuclear factor erythroid 2–related factor 2; RIPK1: Receptor-interacting protein kinase 1; CXCR2: CXC chemokine receptor 2; MSR1: Macrophage scavenger receptor 1; NLRP3: NOD-like receptor protein 3; XBP1: X-box binding protein-1; DC: Dendritic cell; TLR7: Toll-like receptor 7; Tregs: Regulatory T cells; BAFF: B-cell activating factor; NP: Nanoparticle-encapsulated; TAZ: Transcriptional co-activator with PDZ-binding motif; WISP1: WNT1-inducible signaling pathway protein 1; IL-11: Interleukin-11; JAK: Janus kinase; STAT1: Signal transducer and activator of transcription 1; PAR2: Protease-activated receptor-2; VCAM: Vascular cell adhesion molecule; eNOS: Endothelial nitric oxide synthase; LTB4: Leukotriene B4; mPGES: Microsomal prostaglandin E synthases; ARA: Arachidonic acid; CYP4A: Cytochrome P450 4A14; CsA: Cyclosporine A; TRIM5: Tripartite motif protein 5; DDX5: DEAD-box protein 5.

With the rapid development of research techniques, single-cell sequencing and spatial transcriptomics, single-cell proteomics and metabolomics have been widely used and will undoubtedly accelerate the discovery of novel NAFLD/NASH mechanisms and therapeutic targets. Drug libraries enable us to quickly find effective small molecules to target specific pathways identified in the basic research field. Endocrine factors that mediate the crosstalk between the liver and extrahepatic tissues provide an ideal target for developing neutralization antibodies or recombinant proteins for the treatment of NAFLD/NASH. In addition to traditional small molecules, neutralizing antibodies and recombinant proteins, nucleic acid-based therapy has become an attractive approach for the development of drugs for NAFLD/NASH patients. Nucleic acid-based therapeutics include ASOs, siRNAs, miRNAs and mRNAs with modifications to improve their stability, immunogenicity, and diversity[134]. Through nucleic acid-based approaches, therapeutic target genes can be specifically silenced or overexpressed in the liver. As an example, the safety and efficiency of HSD17B13 RNAi[36] and DGAT2 ASOs have been examined in a few clinical trials, which showed a positive impact on liver

Table 2 Novel extrahepatic targets explored by recent basic study

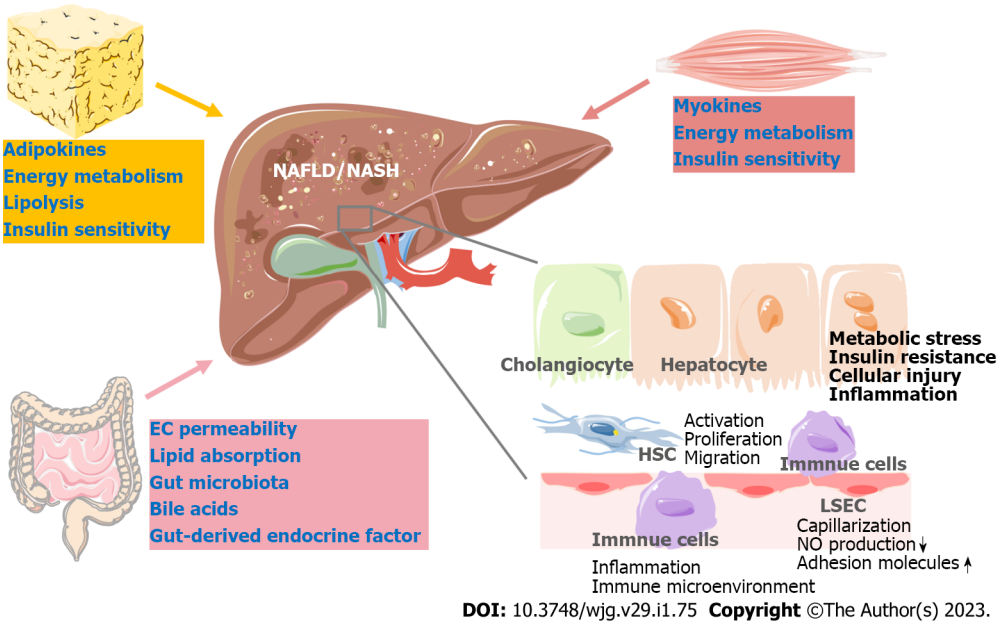
Molecular target	Potential agent	Classification	Function	Effect	Ref.
AMPK	UO-Zn	Small molecule	Improve lipid metabolism	-Lipid accumulation in liver and circulation	[106]
BDK	BT2	Inhibitor	Regulating BCAA metabolism	-Hepatic steatosis -Liver inflammation -Liver fibrosis	[107,108]
HIF-2α (gut)	PT2385	Inhibitor	Decrease intestine-derived ceramide	-Hepatic steatosis -Liver injury	[112]
MYC (gut)	10058-F4	Inhibitor	Decrease intestine-derived ceramide	-Hepatic steatosis -Liver injury -Liver fibrosis	[114]
5-HT(gut)/HTR2A	Sarpogrelate	Antagonist	Inhibit gut-5-HT-liver Htr2α pathway	-Hepatic steatosis	[116]
PPARα (gut)	GW6471	Antagonist	Inhibit fatty acid absorption by intestine	-Hepatic steatosis -Liver inflammation -Liver fibrosis	[117]
MGAT2	BMS-963272	Inhibitor	Regulating fat absorption and liver TG synthesis	-Liver inflammation -Liver fibrosis	[119]
ISM1 (AT)	Recombinant Ism1	Recombinant protein	Suppress lipogenesis in liver	-Hepatic steatosis +Glucose tolerance	[122]
Sparcl1 (AT)	Anti-Sparcl1 antibody	Neutralizing antibody	Decrease CCL2 expression in liver	-Liver inflammation -Liver fibrosis	[123]
Gremlin 1 (AT)	Anti-Gremlin 1 antibody	Neutralizing antibody	Increase insulin sensitivity	-Insulin resistance	[124]
NRG4	hNRG4-Fc	Recombinant protein	Restrain the tumor-prone liver immune microenvironment	-NASH-associated HCC	[125]
SWELL1/LRRC8a (AT)	SN-401	Small molecule	Enhance insulin sensitivity and secretion	+Insulin sensitivity -Hepatic steatosis -Hepatocyte damage	[128]

AT: Adipose tissue; -: Inhibit; +: Stimulate; AMPK: AMP-activated protein kinase; BDK: Branched chain ketoacid dehydrogenase kinase; BCAA: Branched-chain amino acids; HIF-2α: Hypoxia-inducible factor 2α; MYC: Myelocytomatosis oncogene; 5-HT: 5-hydroxytryptamine; PPAR: Peroxisome proliferator-activated receptor; MGAT2: Monoacylglycerol acyltransferase 2; TG: Triglyceride; ISM1: Isthmin-1; Sparcl1: Secreted protein acidic and rich in cysteine-like protein 1; CCL2: C-C motif chemokine ligand 2; NRG4: Neuregulin 4; hNRG4-Fc: Human NRG4 and the Fc domain of immunoglobulin G 1; NASH: Nonalcoholic steatohepatitis; HCC: Hepatocellular carcinoma; UO: Ulvan oligosaccharide; BT2: 3,6-dichlorobrenzo(b)thiophene-2-carboxylic acid.

function and stiffness in patients with NASH[135].

To avoid side effects, efforts have been made to better target the liver or more accurately target specific hepatic cell types by exploring novel drug delivery systems. Based on the hepatocyte-specific expression of asialoglycoprotein receptor, conjugation of GalNAc with nucleic acids or nanoparticles can provide better targeting to hepatocytes. To specifically target HSCs, nanoparticles integrated with vitamin A, cyclic peptides, mannose 6-phosphate, and antibodies against synaptophysin have shown high affinity for HSCs[136,137]. By using these approaches, drugs can be more precisely delivered to HSCs to alleviate NASH-related liver fibrosis. For hepatic macrophages, nanoparticles modified with the phospholipid serine to mimic apoptotic cells can enhance uptake of the nanoparticles by hepatic macrophages[137]. However, challenges remain in targeting other cell types.

Finally, novel preclinical and *in vitro* models are required for drug screening for NASH. A 3D human liver model constructed *via* coculture of hepatocytes with non-parenchymal cells in a 3D collagen matrix has been shown to mimic NASH features when treated with free fatty acids and TNF-α[138]. Liver organoids from pluripotent stem cells or induced pluripotent stem cells also provide unique preclinical platforms to fill the gap between animal studies and clinical trials for therapeutic target validation and



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Figure 1 An overview of intrahepatic and extrahepatic targets for nonalcoholic fatty liver disease/nonalcoholic steatohepatitis treatment. In hepatocytes, strategies for treating nonalcoholic fatty liver disease (NAFLD)/nonalcoholic steatohepatitis (NASH) aim to improve metabolic disturbance and protect hepatocytes from injury. In the liver, the crosstalk network of nonparenchymal cells, including immune cells, hepatic stellate cells, liver sinusoidal endothelial cells and hepatocytes, is an important target for NAFLD/NASH. Outside the liver, the gut, adipose tissue and skeletal muscle interact with the liver *via* various endocrine factors or affect whole-body energy metabolic homeostasis, which has been verified to be an extrahepatic target for NAFLD/NASH treatment. EC: Epithelial cell; HSC: Hepatic stellate cell; LSEC: Liver sinusoidal endothelial cell; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; NO: Nitric oxide.

drug development[139].

FOOTNOTES

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ORCID number: You-Fei Guan 0000-0002-5231-0209.

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MINIREVIEWS

Vibrational spectroscopy – are we close to finding a solution for early pancreatic cancer diagnosis?

Krzysztof Szymoński, Łukasz Chmura, Ewelina Lipiec, Dariusz Adamek

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Krzysztof Szymoński, Łukasz Chmura, Department of Pathomorphology, Jagiellonian University Medical College, Cracow 33-332, Poland

Krzysztof Szymoński, Łukasz Chmura, Dariusz Adamek, Department of Pathomorphology, University Hospital in Cracow, Cracow 31-501, Poland

Ewelina Lipiec, M. Smoluchowski Institute of Physics, Jagiellonian University, Cracow 30-348, Poland

Dariusz Adamek, Department of Neuropathology, Jagiellonian University Medical College, Cracow 33-332, Poland

Corresponding author: Krzysztof Szymoński, MD, Academic Research, Doctor, Researcher, Senior Researcher, Teaching Assistant, Department of Pathomorphology, Jagiellonian University Medical College, Grzegórzecka 16, Cracow 33-332, Poland.
krzysztof.szymonski@uj.edu.pl

Abstract

Pancreatic cancer (PC) is an aggressive and lethal neoplasm, ranking seventh in the world for cancer deaths, with an overall 5-year survival rate of below 10%. The knowledge about PC pathogenesis is rapidly expanding. New aspects of tumor biology, including its molecular and morphological heterogeneity, have been reported to explain the complicated "cross-talk" that occurs between the cancer cells and the tumor stroma or the nature of pancreatic ductal adenocarcinoma-associated neural remodeling. Nevertheless, currently, there are no specific and sensitive diagnosis options for PC. Vibrational spectroscopy (VS) shows a promising role in the development of early diagnosis technology. In this review, we summarize recent reports about improvements in spectroscopic methodologies, briefly explain and highlight the drawbacks of each of them, and discuss available solutions. The important aspects of spectroscopic data evaluation with multivariate analysis and a convolutional neural network methodology are depicted. We conclude by presenting a study design for systemic verification of the VS-based methods in the diagnosis of PC.

Key Words: Spectroscopic cancer diagnosis; Raman spectroscopy; Pancreatic cancer diagnosis; DNA methylation; Liquid biopsy biomarkers; Convolutional neural networks

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Core Tip: Vibrational spectroscopy (VS) may become a major player in the development of early diagnosis technology for pancreatic cancer. As with every technique, VS has promising attributes as well as drawbacks. We summarize recent reports about improvements in spectroscopic methodologies, briefly explain and highlight the drawbacks of each of them, and discuss available solutions. Additionally, the important aspects of spectroscopic data evaluation with multivariate analysis and a convolutional neural network methodology are depicted.

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INTRODUCTION

Pancreatic cancer (PC) is a very aggressive and lethal neoplasm, ranking seventh in the world for cancer deaths[1]. In 2020 there was an estimated number of 466003 new PC-related deaths worldwide[2]. Despite a better understanding of the nature of pancreatic tumors, 5-year survival rates have not improved and remain below 10%. Late-stage disease at diagnosis is a significant issue that contributes to poor overall survival rates. Another prognostically relevant, yet complex factor is related to the morphological and molecular heterogeneity of the tumor cells and the surrounding stroma. On one hand, the need for proper pathological evaluation with a detailed prognostic assessment is required, on the other, more importantly, the variability of PC leads to chemoresistance[3]. Moreover, differentiating PC from large tumors of the ampulla of Vater remains challenging[4]. Our previous article highlighted recent trends in PC pathology and research[3]. Vibrational spectroscopy (VS)-based methods will play prominent roles in the early diagnosis of PC (Figure 1). Detailed studies regarding the molecular nature of PC are required to reveal novel early and precise diagnostic technologies, thus improving survival rates.

EARLY DIAGNOSIS OF PC

The lack of specific and sensitive early diagnostic options for PC screening results in late-stage disease at the time of diagnosis and is one of the reasons for the poor overall PC survival rates. Out of the available options, serum antigen levels, such as carbohydrate antigen 19-9, are insufficient because of poor specificity and sensitivity for malignancy detection[5,6]. Some studies highlighted the usefulness of measuring interleukin-6 serum levels to differentiate pancreatic ductal adenocarcinoma (PDAC) from chronic or acute pancreatitis[7-9]. Recently, leukemia inhibitory factor was reported to be a promising serum biomarker of pancreatic malignancy[10], a monitoring indicator of treatment response[11-14], and a predictor of metastatic disease in PDAC patients[15].

LIQUID BIOPSY BIOMARKERS

Circulating tumor DNA

Recently, some new circulating biomarkers, particularly those associated with early-stage disease, have been intensely studied. These include a group of tumor nucleic acids in the patient's blood serum, such as circulating tumor DNA (ctDNA), cell-free DNA, cell-free RNA, circulating tumor cells, or extracellular vesicles (EVs) (Figure 2)[16]. Aberrant methylation markers specific for certain malignancies, including PDAC, were previously detected using genetic methods, such as next-generation sequencing and droplet digital polymerase chain reaction[17-20], but the sensitivity of these methods is still low[20].

EVs

Both tumor and healthy cells release lipid-membrane vesicles, termed “extracellular vesicles”, into the bloodstream. EVs include oncosomes, apoptotic bodies, microvesicles, and exosomes of different sizes and biogenesis[21,22]. They have an established role in cancer cell communication and metastasis[23,24]. EVs are released in a passive mode (apoptotic bodies) from dying cells (apoptosis, necrosis) or in an active mode (microvesicles, exosomes) from living cells[25]. They make up a large part of the ctDNA (single-strand DNA and double-strand DNA) in a liquid biopsy, but also carry other biomarkers, such as tumor protein antigens[26,27], or microRNA[28,29]. The cargo within an EV creates a unique spectro-

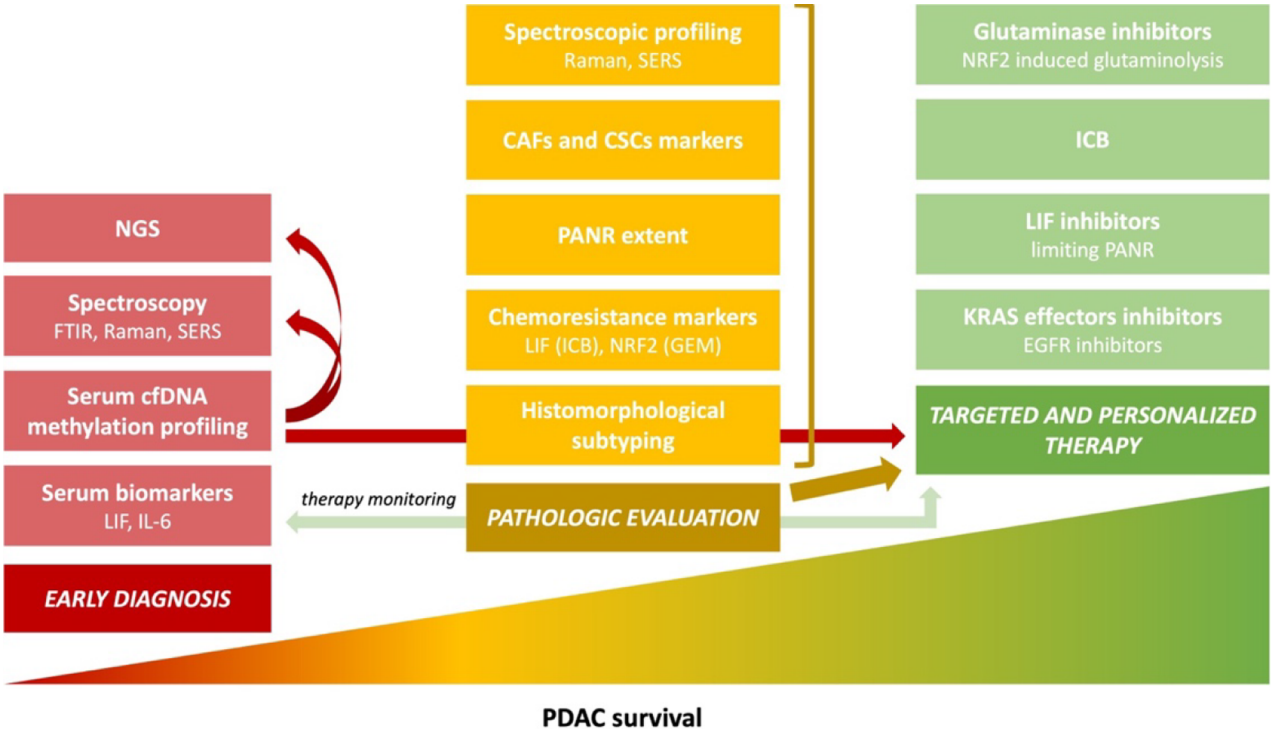


Figure 1 Main trends in pancreatic cancer pathology and research aim to improve survival[3]. Poor pancreatic ductal adenocarcinoma (PDAC) prognosis for a patient is multifactorial, and the lack of sensitive and specific early diagnostic methods is one of the reasons. Another is the resistance to available therapeutic options, which is caused, among others, by the tumor's molecular and morphological heterogeneity. Detailed pathological reporting is crucial for targeted and personalized therapy. The development of new diagnostic methods, combined with a proper pathologic evaluation and spectroscopic profiling, lead to effective treatment and supposedly will increase PDAC patients' survival rates; Adapted with permission from[3]. Citation: Szymoński K, Milian-Ciesielska K, Lipiec E, Adamek D. Current Pathology Model of Pancreatic Cancer. *Cancers (Basel)* 2022; 14: 2321. Copyright© The Authors 2020. Published by MDPI. The image may be redistributed without special permissions–source: https://www.mdpi.com/openaccess. PDAC: Pancreatic ductal adenocarcinoma; LIF: Leukemia inhibitory factor; IL-6: Interleukin-6; cfDNA: Cell-free DNA; FTIR: Fourier transform infrared spectroscopy; Raman: Raman spectroscopy; SERS: Surface-enhanced Raman spectroscopy; NGS: Next-generation sequencing; ICB: Immune checkpoint blockers; NRF2: Nuclear factor-erythroid factor 2-related factor 2; GEM: Gemcitabine; PANR: PDAC-associated neural remodeling; CAFs: Cancer-associated fibroblasts; CSCs: Cancer stem cells; EGFR: Epithelial growth factor receptor.

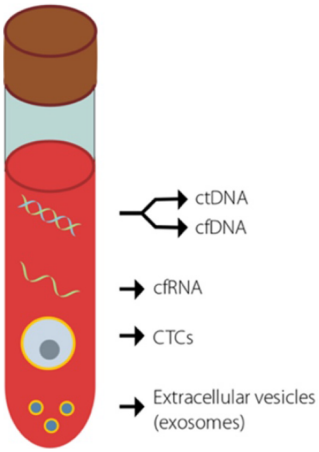


Figure 2 Potential cancer biomarkers that are detectable using liquid biopsies[16]. Key biomarkers that are currently used in an attempt to detect early-stage cancer. Adapted with permission from[16]. Citation: Jaworski JJ, Morgan RD, Sivakumar S. Circulating Cell-Free Tumour DNA for Early Detection of Pancreatic Cancer. *Cancers (Basel)* 2020; 12: 3704. Copyright© The Authors 2020. Published by MDPI. The image may be redistributed without special permissions–source: https://www.mdpi.com/openaccess. ctDNA: Circulating tumor DNA; cfDNA: Cell-free DNA; cfRNA: Cell-free RNA; CTCs: Circulating tumor cells.

scopic fingerprint specific for certain tumors, including PDAC. The molecular characteristics that were obtained using VS, when combined with the use of convolutional neural networks (CNN), can be specified to pinpoint valuable information from the general all-in-one data, thus creating a tool for successful diagnosis.

DNA methylation

DNA methylation plays an important role in the regulation of gene expression, and cellular differentiation. Aberrant methylation is associated with the development and progression of various cancers[16,30,31]. Moreover, these specific aberrations may act as cancer biomarkers, enabling early diagnosis. Standard methylation status evaluation techniques include bisulfite conversion assay, the sequencing or melting curve analysis, restricted enzymes-based assay, and affinity capture using methylated DNA binding proteins[31]. Some of these methods suffer from a laborious workflow and false positives. Others require specific reagents such as enzymes and binding proteins, which are costly and time-consuming. Therefore, we will discuss the usefulness of label-free spectroscopic methods, such as surface-enhanced Raman spectroscopy (SERS), in DNA methylation status detection, and we will address potential issues that arise when using these techniques.

PDAC HETEROGENEITY AND CHEMORESISTANCE

PC is well known to be very heterogeneous in molecular and morphological phenotype. It is one of the reasons, aside from the lack of adequate early diagnosis methods, for patients' poor prognosis, because current treatment options do not consider tumor heterogeneity and thus give insufficient results[32,33]. When designing studies based on PC diagnosis, one must differentiate the results concerning the histomorphological type of the tumor. This distinction is crucial for patient care, due to different molecular pathways governing the development and evolution of these tumors, as well as the prognosis assessment and potential treatment options[3]. In our previous study, we demonstrated that Raman spectroscopy (RS) is capable of detecting ampullary cancer in pancreatic tumor tissue slides[34]. A subsequent step could then differentiate between three groups of pancreatic tumors, specifically conventional PDAC (cPDAC), PDAC derived from intraductal papillary mucinous neoplasm (IPMC), and ampulla of Vater adenocarcinoma (AVAC). cPDAC is the most common form of PC developing *via* pancreatic intraepithelial neoplasia. It arises in the ductal epithelium localized in "normal" pancreatic tissue, sometimes with signs of chronic pancreatitis, or is combined with so-called "acinar-to-ductal metaplasia" regions. The KRAS mutation is the initiating event in this pathway of carcinogenesis[3]. IPMCs are carcinomas that arise from intraductal papillary mucinous neoplasms (IPMNs), cystic tumors that develop in the main or peripheral branches of pancreatic ducts. Guanine nucleotide-binding protein, alpha stimulating activity polypeptide proto-oncogene mutation, which is not found besides pancreatic tumors, plays a significant role in IPMN development[3]. The third relevant group, AVAC, is a cancer of the duodenal ampulla of Vater. AVAC tumors usually grow with large diameters and deeply infiltrate the pancreatic tissue. The histomorphological similarities of AVAC and cPDAC tumors often make them hard to distinguish from each other. Moreover, these tumors are treated clinically and diagnostically in the same way, but the latest reports suggest that they differ regarding the incidence of various prognostic factors, such as tumor differentiation, perineural invasion, venous invasion, or lymph node involvement[35]. The early occurrence of bile duct obstruction symptoms in IPMC or AVAC enables earlier diagnosis and thus may lead to a better prognosis. However, the reports supporting this are ambiguous[3]. Further subgrouping of PC tumors into morphologically distinct entities, such as large duct (cystic papillary), foamy glands, clear cell, adenosquamous, vacuolated-cell, or colloid, which are described in detail elsewhere[3], may benefit patients and clinicians, because of the different prognosis of some of these groups.

Recently, Mukhopadhyay *et al*[36] showed that the nuclear factor-erythroid 2–related factor 2 (NRF2) was responsible for gemcitabine chemoresistance, and the NRF2 expression level in PDAC tissues correlated with poor patient outcome. Another study by Patzak *et al*[37] described cytosolic 5′-nucleotidase 1A (NT5C1A) as a mediator of this resistance by reducing intracellular gemcitabine metabolites and limiting its efficacy. Gemcitabine is a standard chemotherapeutic agent for PDAC. The tumor's resistance to the therapy is among the main reasons for the drastically low 5-year survival rates of PC. Thus, recognizing this chemoresistance is of great importance. The utilization of VS is a step closer in this direction. VS may prove to be beneficial for identifying chemoresistant pancreatic tumors. More studies are required to evaluate NRF2 or/and NT5C1A expression levels in PDAC tissues, and compare them with spectroscopic data to identify spectral markers that correlate with gemcitabine chemoresistance. These studies will help select patients who might benefit from gemcitabine therapy.

Molecular spectroscopy can obtain all the information about the studied sample with a single measurement. There is no need for special labeling or selecting areas of interest that other genetic or biochemical methods may require. Analyzing spectral data plays a pivotal role. All data is ready for interpretation and provides information about the tumor, such as its subtype, differentiation level, specific chemoresistance, and other tumor-specific poor prognostic factors (hepatocyte nuclear factor-1B expression, or cancer stem cells). Whether or not we can decipher this information will determine the efficacy of this methodology in diagnostics.

MOLECULAR SPECTROSCOPY

Molecular VS was confirmed to play an important role in the characterization of the chemical structure and composition of malignant tissues[38-41] and the analysis of human blood serum[42]. Due to high chemical selectivity, RS and infrared spectroscopy (IR) might evolve into efficient pancreatic malignancy screening tools[43]. These methodologies provide information about various biologically significant molecules and functional groups in a tumor including phospholipids and triglycerides, proteins, nucleic acids, phosphates, and carbohydrates. Electromagnetic radiation (photons) can be absorbed and/or scattered by a sample because the energy of phonon excitations and vibrations, as well as oscillations of functional groups in molecules correspond to the energy in the infrared region of the electromagnetic radiation spectrum. Therefore, the presence of functional groups in the analyte can be detected *via* interaction with light, providing information about the molecular structure and composition of the investigated sample. IR and RS take advantage of absorption and inelastic scattering, respectively. The results obtained using these methods indicate differences in metabolic pathways typical for various neoplasms. The main advantage of the molecular spectroscopic approach is collecting information about samples in a label-free and noninvasive manner. The potential of spectroscopic methods has not yet been fully explored in the investigation of PC. However, preliminary results are promising[34,44].

Raman hyperspectral mapping

RS is a VS method that can deliver a detailed molecular fingerprint of a studied sample almost real-time, without the need for special labeling. A technique called Raman hyperspectral mapping (RHM) provides high-resolution imaging at a relatively low cost compared to other well-established medical imaging techniques, such as magnetic resonance imaging (MRI)[43]. RHM relies on multiple measurements of adjacent "pixels" of tissue and integrating the resulting spectra into a single map image. Such imaging allows the selection of points of interest in the studied sample and precise distinction between particular tissue elements, such as cancer cells (with nuclei and cytoplasm) or the stroma compartment (Figure 3).

The RHM methodology of tissue samples usually involves slicing 2.5 μm thick tissue sections with a microtome from a standard formalin-fixed paraffin-embedded tissue block. Because glass gives substantial interference in the Raman readings, for the slide mounting, special calcium fluoride (CaF_2) windows are used for the slide mounting instead. Another important aspect of RHM is the selection of the region of interest which is usually a part of the cancerous glands or stroma compartment. This should be completed by an experienced pathologist. Subsequently, a complete paraffin removal ought to be conducted involving a 12-h xylene bath and graded ethanol rehydration. On such preprocessed tissue slides, the Raman measurements can be performed. This methodology was already described in our previous paper on ampullary cancer detection with RHM[34].

RS is accurate, and the information obtained is characterized by good resolution. Nevertheless, RS requires a sophisticated methodology and equipment because the Raman effect is very weak in nature [43,45]. Another drawback of RS is the substantial sample pre-processing, manual selection of points of interest, and further data analysis.

SERS

To augment the Raman signal strength, another method called SERS is used[43,45]. SERS is a label-free, ultrasensitive tool, capable of DNA methylation analysis. SERS utilizes the same physical phenomenon as RS (Raman effect), but the effect is significantly enhanced using specially synthesized plasmonic nanoparticles[46] into which the molecules (such as DNA) are absorbed [31]. Furthermore, SERS is efficient for liquid biopsy measurements, and it does not require special labeling. However, the production of nanoparticles requires an experienced team and a proper methodology.

The unique ability for such sensitivity of SERS is achieved *via* the use of plasmonic nanostructures (SERS substrates), between which so-called "hot spots" are localized. The traditional methodology of SERS measurements involved mixing the sample with gold (Au) nanoparticles (20-50 nm diameter). One method of SERS substrate production prepares Au nanoparticles *via* chemical reduction of tetrachloroauric (III) acid using trisodium citrate under specific reaction conditions according to the procedure described by Frens[46]. However, this synthesis method is characterized by random and nonuniform hot spot distribution, which leads to the poor reproducibility of SERS[31,47,48]. Another issue, especially regarding DNA methylation studies, is the difficulty distinguishing between DNA methylation signals and the adjacent nucleotide signals due to their similarity[49]. Additionally, surfactants and/or capping agents can affect signal purity[50]. Overcoming these issues is crucial to utilizing SERS for diagnostics largely based on DNA methylation analysis. To achieve this, a methodology of proper SERS substrate development is required. Such a substrate is characterized by a large Raman enhancement, regularly arranged hot spots, and an open and easily accessible surface topology[31]. Luo *et al*[31] proposed the use of a plasmonic gold nanohole array (PGNA) as a SERS substrate. Originally, the authors described the use of electron beam lithography (EBL) for the PGNA substrates production, but a focused ion beam (FIB) might be even better, due to its higher resolution [51]. Both EBL and FIB can be used to obtain a periodic matrix of holes (plasmonic nano-holes array) in a

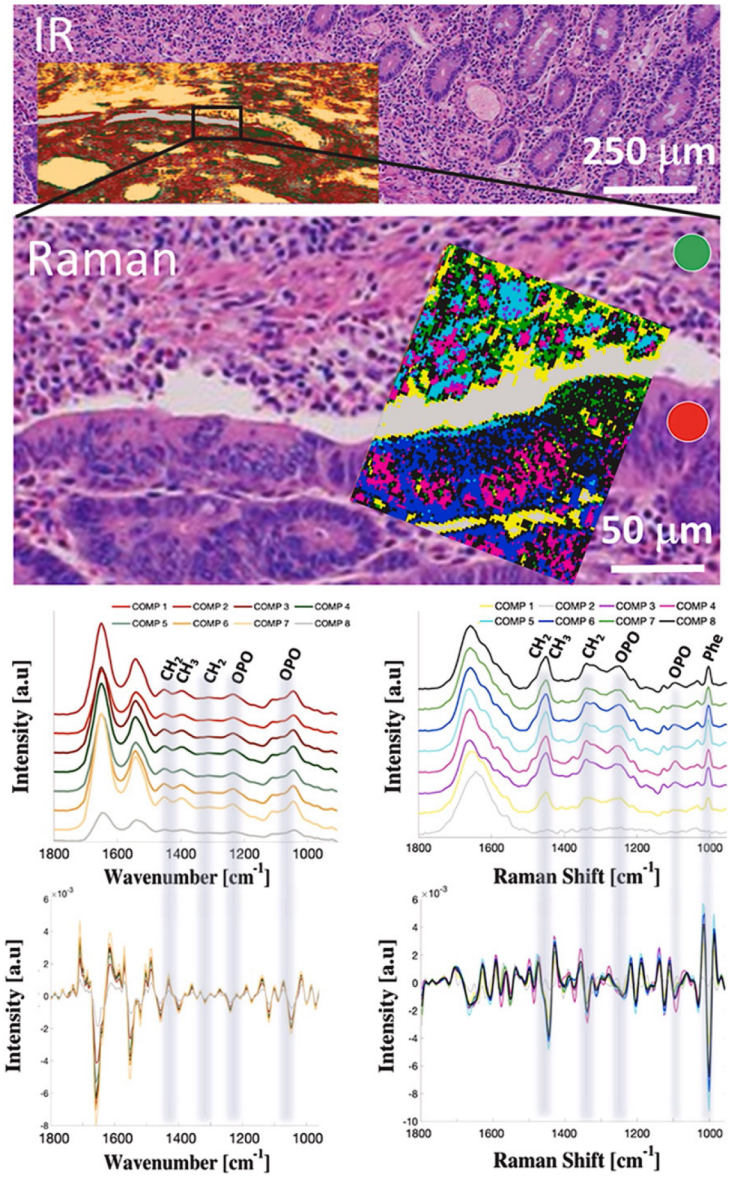


Figure 3 Spectroscopic mapping of ampullary adenocarcinoma[34]. A hematoxylin-eosin slide image of ampullary cancer tissue with superimposed Fourier transform infrared spectroscopy and Raman hyperspectral maps treated with hierarchical cluster analysis and averaged spectra with corresponding second derivatives from each cluster. Spectroscopic maps cover both cancerous (red circle) and noncancerous–stroma (green circle) tissue fragments. Adapted with permission from[34]. Citation: Szymoński K, Lipiec E, Sofińska K, Skirlińska-Nosek K, Milian-Ciesielska K, Szpor J, Czaja M, Seweryn S, Wilkosz N, Birarda G, Piccirilli F, Vaccari L, Szymoński M. Spectroscopic screening of pancreatic cancer. *Clin Spect* 2021; 3: 100016. Copyright© The Authors 2020. Published by ELSEVIER.

Au layer evaporated onto an atomically flat non-plasmonic substrate[31] (Figure 4).

SERS is characterized by a large amount of work required for substrate production, but the sample pre-processing is minimal. Raw blood serum samples can be analyzed, without requiring preselection by a specialist. This is a valuable asset in the search for a diagnostic option.

Attenuated total reflection Fourier transform infrared spectroscopy

Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) is a complementary method to SERS and RS, which measures absorption as an infrared spectrum of a biochemical fingerprint. It is widely accessible, inexpensive, and easy to use and implement, while still providing substantial information about the sample being analyzed. ATR-FTIR is characterized by high sensitivity in biomedical diagnostics[52] and can detect spectral markers of many pathologies in physiological liquids such as saliva or blood serum. ATR-FTIR was used to detect lung[53], bile duct[54], ovary[55, 56], breast[57], and brain[58,59] tumors in the blood serum samples. In a study by Butler *et al*[59], the team described the methodology of using ATR-FTIR in an early screening of brain tumor patients with sensitivity and specificity of 93.2% and 92.8%, respectively. The high sensitivity of ATR-FTIR can be achieved due to the design of the instrumental set-up. In the ATR-FTIR, a sample droplet is placed on an internal reflection element (IRE), also called the ATR crystal. IREs with high refractive indexes, such

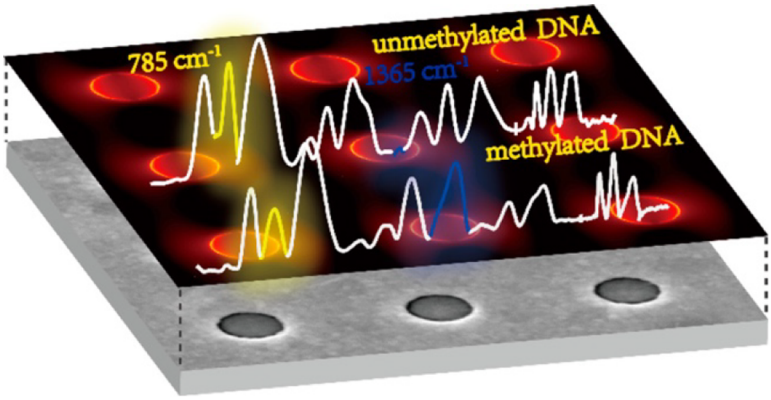


Figure 4 A schematic of a plasmonic gold nanohole array as a surface-enhanced Raman spectroscopy-active substrate used in the detection of DNA methylation[31]. Citation: Luo X, Xing Y, Galvan DD, Zheng E, Wu P, Cai C, Yu Q. Plasmonic Gold Nanohole Array for Surface-Enhanced Raman Scattering Detection of DNA Methylation. *ACS Sens* 2019; 4: 1534–1542. Copyright© The Authors 2020. Published by American Chemical Society.

as diamond, zinc selenide, germanium, or silicon are used. The incident IR beam is directed through the IRE and the resultant evanescent wave extends beyond the ATR crystal surface and penetrates the sample. As with SERS, there is no need for sample pre-processing or special labeling. No additional substrate production is required as well. However, achieving sensitive information about the samples, and their interpretation remains challenging. Employing CNNs to analyze ATR-FTIR data is a viable solution that has already shown success[59].

Molecular spectroscopy in diagnostics

Multiple studies have shown the usefulness of VS in the detection of DNA methylation status of cells, as well as other diagnostically important factors. For example, DNA methylation aberrations were successfully detected using SERS[31,49,60-62], and FTIR[63-69] methods. Recently, in an interesting study by Ho *et al*[70], the authors successfully utilized RS and deep neural networking that enabled culture-free serum bacteria identification and antibiotic susceptibility testing with about 97% accuracy. In other studies[71,72], RS and SERS were used to detect previously untraceable concentrations of biomarkers (matrix metalloproteinase 7, and mucin 4) in the serum of PDAC patients.

To date, no report has combined detecting aberrant DNA methylation markers of PDAC, obtained by analyzing patients' serum with the use of VS methods, specifically the ATR-FTIR combined with CNN classification. Although partial results are available and show promise, additional investigations are needed to support the combination of VS and CNN for PDAC detection.

Residual disease monitoring

Currently, there are no efficient methods of patient monitoring for minimal residual disease (MRD) in PC. Generally, postoperative surveillance methods, including monitoring of clinical symptoms, blood tumor markers, and computed tomography or MRI are used, but these methods lack sensitivity and specificity for MRD[73]. Recently, ctDNA detection using genetic methods in a liquid biopsy was highly advocated for MRD monitoring[73-76]. VS could be a better option for MRD monitoring compared to genetic mutation detection, because of VS's ability to identify DNA methylation aberrations[20]. However, there are currently no data supporting this. Notably, as highlighted by Henriksen *et al*[77], surgical trauma elevates the serum ctDNA levels up to 4 wk after the surgery. This should be taken into consideration when designing a MRD monitoring study using liquid biopsy analysis.

MULTIVARIATE DATA ANALYSIS

In spectroscopic data evaluation, it is very important to draw proper conclusions. Therefore, various methods of multivariate analysis are used to help with data interpretation[78]. Hyperspectral mapping with K-means clustering and principal component analysis (PCA) is commonly performed to explore spectral variation[79-81]. Pre-processing of the spectroscopic data involves cosmic rays removal, baseline correction, and smoothing (adaptive multi-round smoothing based on the Savitzky-Golay filter). Minimal necessary operations are performed to explore marker bands of significant aspects, such as DNA methylation while preventing the loss of important spectral information. Multivariate analysis is carried out to reduce data dimensionality and extract the most important parameters from the acquired information. Some of these methods are briefly described below.

Unsupervised hierarchical cluster analysis is a clustering algorithm designed to group the obtained spectra or to produce false-colored maps based on spectral similarity and variability.

PCA is based on a linear transformation of the data to a new space described by orthogonal axes, the so-called principal components. The most significant results are the "score" values, which represent the data in multidimensional space corresponding to the principal components, and the loading values, which identify the variables causing the data separation according to their influence on the scores. Additionally, the results of the PCA are complemented by the explained variance. The explained variance gives the percentage of the total variance of the original data set, which is explained by a certain principal component.

Partial least squares regression (PLSR) involves a linear transition of numerous original descriptors to a new variable space based on a small number of orthogonal factors (latent variables). In other words, PLSR allows the construction of predictive models when the factors are highly collinear. This analysis estimates unobservable variables as exact linear combinations of their empirical indicators. The estimated proxies are treated as substitutes for the latent variables. The selected case values capture most of the independent variables' variance. This variance is used for predicting the dependent variable.

Non-negative matrix factorization (NMF) is a useful tool for the analysis of high-dimensional data. Besides detecting a compressed representation, NMF provides insights into the structure and features of the given data by extracting easily interpretable factors. With the use of NMF, basic spectral components for proteins, lipids, phospholipids, or nucleic acids can be compared.

All of these methods depend on human decision-making, although minimal. As a result, these methods might disturb and lose seemingly irrelevant data. To overcome this and enhance the sensitivity and specificity of spectral data interpretation, deep neural networks are used.

CNNs

RS is characterized by a very low strength of the measured effect (Raman effect) and is thus very sensitive to distortion factors, such as fluorescence, thermal noise, the quality of the measuring equipment, and research team experience. Removing noise artifacts requires various pre-processing methods on acquired spectra (*i.e.*, cosmic rays removal, baseline correction, and smoothing). All of these depend on human input and definition. On one hand, this prevents automation, but on the other, some seemingly irrelevant data might be lost during the pre-processing. The successful use of CNNs in spectroscopic data evaluation and classification was shown in multiple studies[82,83] including those using RS[84], SERS[70,85], and ATR-FTIR[86]. As a source, the CNN is fed with raw spectral data, without human interaction-related pre-processing. This approach gives better results in classification than conventional methods[82] and makes the methodology more universal. CNN objectives should be clear and simple. Similarly, the proper selection of training data is of great importance. For each objective, different training sets should be created. All spectral results from each group are split (2:1) to form the training (two-thirds of data) and testing (one-third of data) datasets. Training datasets should include positive and negative cases, preferably with many variations. For example, when designing a training dataset for the CNN that will decide whether the results are from a malignant pancreatic tumor or not from a malignant pancreatic tumor, one might include cases from PC, but as a negative control also include malignancies of other sites (*i.e.*, colorectal or breast carcinomas), and benign pancreatic entities (*i.e.*, IPMN, mucinous cystic neoplasm, or groove pancreatitis).

There are some issues related to utilizing neural networks (NNs) with multiple layers (deep NNs), specifically very deep NNs. One obstacle is the vanishing/exploding gradient problem. A training process of a NN in simplest words usually involves updating the "weights" of the algorithm to better cope with the problem, that the NN is exposed to. A great benefit of a CNN is that it extracts features of the task on its own. Feature extraction is done using an optimization algorithm, such as "gradient descent", which simply finds values of a function's parameters to minimize the cost function. If gradients that update the weights shrink, the weights are no longer updated, and the learning stops. This is called a vanishing gradient problem. Similarly, if gradients grow, weights do not update reasonably, and the learning becomes unstable, resulting in the exploding gradient problem. The so-called "skip connection" technique is utilized to overcome the vanishing/exploding gradient problem [83,87]. This is the basis for a Residual Network CNN architecture[87]. Another issue of CNNs is data overfitting. When a model used to train the CNN is very complex, and there is a limited amount of learning data available, the CNN learns to know the training dataset well, but performs poorly against any new data (*i.e.*, validating/testing dataset or the implementation data). To improve this shortcoming, proper data augmentation techniques might be needed. Usually, in spectral data analysis, augmentation requires making additional artificial spectra by small spectral shifting, expanding spectral range, adding Gaussian noise, or superimposing the spectra for each real result[83].

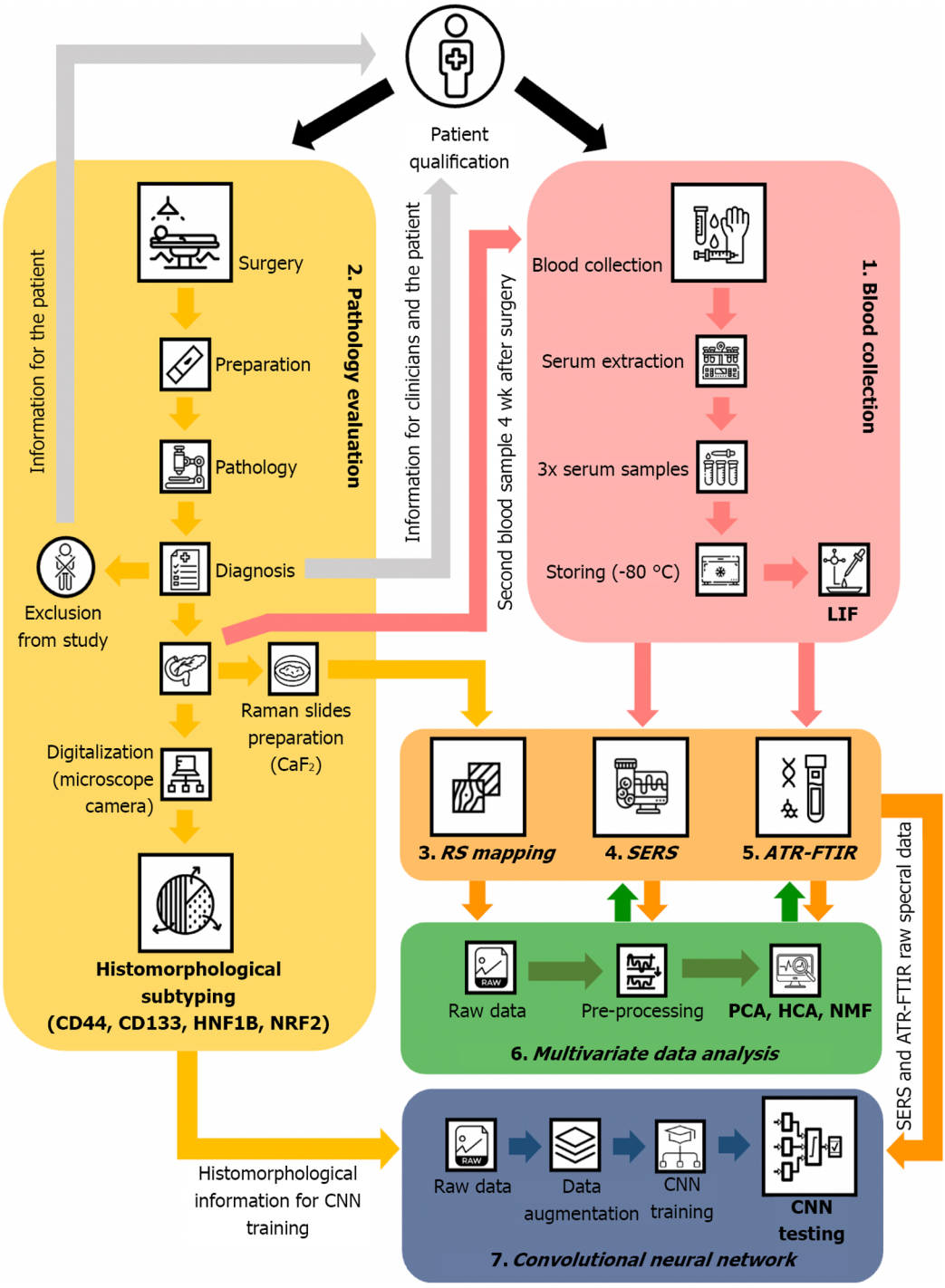
Table 1 Drawbacks of spectroscopic-related methodologies, and proposed solutions

Methodology	Issue	Solution
Raman spectroscopy (random measurement points selection)	(1) Not differentiating the results with cancer subtype leads to mixed results; and (2) Mixing measurements of cancer cells or tumor stroma with pancreatitis, inflammation, necrosis, colloid, *etc*	(1) Results should be analyzed with regard to cancer subtype; (2) Raman hyperspectral mapping allows the selection of specific points of interest, whether cancer cells or tumor stroma areas; and (3) The selection should be done by an experienced pancreatic pathologist
SERS	(1) Poor reproducibility, due to random and nonuniform hot spots distribution in nanostructures production[31,47,48]; (2) The distinction between methylation signals and those from adjacent nucleotides is difficult, because of their similarity[49]; and (3) Signal purity is affected by the use of surfactants and/or capping agents[50]	(1) PGNA as a SERS substrate; and (2) The use of a FIB to obtain a periodic matrix of holes (plasmonic nano-holes array) in a gold layer evaporated on an atomically flat non-plasmonic substrate[31]
ATR-FTIR	(1) Low sensitivity and specificity; (2) Human-dependent pre-processing and analysis of spectral data; and (3) Not yet confirmed in pancreatic cancer diagnosis	To increase the sensitivity and specificity, and to limit the human intervention, convolutional neural networks are used
Multivariate data analysis	(1) Results might be disturbed by the human-dependant actions and seemingly irrelevant data will be lost; and (2) Losing data lowers the sensitivity and specificity of the method	Using of convolutional neural networks, which are fed with raw spectral data
CNN	(1) Very deep neural networks are characterized by a vanishing/exploding gradient problem; and (2) Overfitting of the CNN trained on a limited amount of data	(1) The use of ResNet CNN architecture, with the use of so-called "skip connections"; and (2) Proper data augmentation to prevent overfitting and sensitize the CNN to deal with various "scenarios"

PGNA: Plasmonic gold nanohole array; FIB: Focused ion beam; SERS: Surface-enhanced Raman spectroscopy; ResNet: Residual Network; CNN: Convolutional neural networks; ATR-FTIR: Attenuated total reflection Fourier transform infrared spectroscopy.

CONCLUSION

When designing a study investigating VS methods in PC diagnosis, it is important to bear in mind a couple of aspects. First, the study is required to address all the drawbacks of RS, SERS, and ATR-FTIR methodology, described briefly above. Another crucial issue is the proper cooperation of multidisciplinary teams, including medical specialists, such as pathologists and clinicians, and spectroscopic specialists, such as physicists and chemists. For example, comparing PC cases with pancreatic neuroendocrine neoplasms is not reasonable because the malignancies represent different entities with very distinct pathways governing their initiation and progression, as much as patient survival. When dealing with PC, detailed knowledge of the tumor's molecular and pathological nature is required. In particular, one should interpret the results of measurements in the context of PC subtypes and some other prognostic factors[3]. Proper analysis of measured spectroscopic data is impartant. For example, when conducting a RS on complex tissue samples, such as PC sections, the random spots of measurements will yield ambiguous results. The spectral data might be disturbed by numerous interfering phenomena, such as inflammation, tumor necrosis, or fibrosis. Additionally, the cytoplasmic and nuclear regions of cancer cells significantly differ as well. Interpretation of the PC stroma compartment, including the complicated cancer-stroma "cross-talk", is another aspect to address[3]. Careful selection of regions of interest is important too. This should be done by a specialized pancreatic pathologist familiar with the spectroscopic methodology. Finally, in the search for a PC diagnostic tool, we look for universality and automation. Thus, the interpretation of spectral data obtained from liquid biopsy, which relies on human-dependent pre-processing is not a good path to follow. CNNs are invaluable here, but proper design and training is the key to success. With these aims, we designed a study that will comprehensively evaluate the potential of VS methods used in diagnosing PC, by systemically evaluating liquid biopsy samples (Figure 5). In conclusion, VS seems to be leading the way in the race, with most of the methodology drawbacks resolved, at least partially (Table 1).



DOI: 10.3748/wjg.v29.i1.96 **Copyright** ©The Author(s) 2023.

Figure 5 A Designed study regarding the diagnostic potential of vibrational spectroscopic methods used for diagnosing pancreatic cancer by systemically evaluating liquid biopsy samples. LIF: Leukemia inhibitory factor; RS: Raman spectroscopy; SERS: Surface-enhanced Raman spectroscopy; ATR-FTIR: Attenuated total reflection Fourier transform infrared spectroscopy; CNN: Convolutional neural network; NRF2: Nuclear factor-erythroid 2–related factor 2; HNF1B: Hepatocyte nuclear factor-1B; PCA: Principal component analysis; HCA: Hierarchical clustering analysis; NMF: Non-negative matrix factorization.

FOOTNOTES

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Country/Territory of origin: Poland

ORCID number: Krzysztof Szymoński 0000-0002-6127-1918; Łukasz Chmura 0000-0003-2768-0643; Ewelina Lipiec 0000-0003-2946-7387; Dariusz Adamek 0000-0002-5570-2259.

Corresponding Author's Membership in Professional Societies: European Society of Pathology, No. 0000030740.

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MINIREVIEWS

Unveiling the biological role of sphingosine-1-phosphate receptor modulators in inflammatory bowel diseases

Evanthia Tourkochristou, Athanasia Mouzaki, Christos Triantos

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Evanthia Tourkochristou, Christos Triantos, Division of Gastroenterology, Department of Internal Medicine, Medical School, University of Patras, Patras 26504, Greece

Athanasia Mouzaki, Division of Hematology, Department of Internal Medicine, Medical School, University of Patras, Patras 26504, Greece

Corresponding author: Christos Triantos, FAASLD, MD, PhD, Assistant Professor, Division of Gastroenterology, Department of Internal Medicine, Medical School, University of Patras, Rion, Patras 26504, Greece. chtriantos@hotmail.com

Abstract

Inflammatory bowel disease (IBD) is chronic inflammation of the gastrointestinal tract that has a high epidemiological prevalence worldwide. The increasing disease burden worldwide, lack of response to current biologic therapeutics, and treatment-related immunogenicity have led to major concerns regarding the clinical management of IBD patients and treatment efficacy. Understanding disease pathogenesis and disease-related molecular mechanisms is the most important goal in developing new and effective therapeutics. Sphingosine-1-phosphate (S1P) receptor (S1PR) modulators form a class of oral small molecule drugs currently in clinical development for IBD have shown promising effects on disease improvement. S1P is a sphingosine-derived phospholipid that acts by binding to its receptor S1PR and is involved in the regulation of several biological processes including cell survival, differentiation, migration, proliferation, immune response, and lymphocyte trafficking. T lymphocytes play an important role in regulating inflammatory responses. In inflamed IBD tissue, an imbalance between T helper (Th) and regulatory T lymphocytes and Th cytokine levels was found. The S1P/S1PR signaling axis and metabolism have been linked to inflammatory responses in IBD. S1P modulators targeting S1PRs and S1P metabolism have been developed and shown to regulate inflammatory responses by affecting lymphocyte trafficking, lymphocyte number, lymphocyte activity, cytokine production, and contributing to gut barrier function.

Key Words: Inflammatory bowel disease; Sphingosine-1-phosphate; Intestinal inflammation; T helper 1/T helper 17; Sphingosine 1 phosphate; Modulators; Immune responses

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Core Tip: Recent literature has highlighted the use of novel oral small molecules, so-called sphingosine-1-phosphate (S1P) receptor modulators, in the therapeutic treatment of inflammatory bowel disease (IBD). Reviews and clinical trials have reported the safety profile and role of S1P modulators in alleviating IBD, but little information is available on their biological function. This is a comprehensive mini-review describing key biological mechanisms beyond the activity of S1P modulators reported in preclinical and clinical studies. The data from this study will contribute to the research field of developing therapeutic strategies in IBD based on the pathogenic biological background.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammation of the gastrointestinal tract that includes two main forms: ulcerative colitis (UC) and Crohn's disease (CD). IBD is thought to be mediated by dysregulation of the immune system, and its high epidemiological prevalence worldwide has led to great concern regarding the treatment and therapeutic management of patients[1]. Although current therapeutic agents have led to significant improvements in IBD in recent years, there is still a large percentage of patients who do not respond to these agents[2], and high immunogenicity has also been associated with monoclonal antibodies[3]. Sphingosine-1-phosphate (S1P) receptor modulators are novel oral small molecule drugs that have better prospects in terms of route of administration, pharmacokinetic properties, immunogenicity, manufacturing and cost compared to other biologic agents in IBD [4]. S1P is a highly bioactive molecule that regulates important biological processes such as inflammation, immune cell transport, and cell growth and transformation[5]. S1P level creates a gradient between blood and tissues, which contributes to the recruitment of immune cells and inflammatory mediators in tissues. S1P exerts its biological function by activating specific S1P receptors expressed by different cell types, initiating a signaling cascade that modulates lymphocyte migration, endothelial permeability, angiogenesis, cell proliferation, cell survival, apoptosis, and differentiation[6]. The components of S1P metabolism and S1P signaling have been associated with the regulation of immune responses and inflammation-related pathologies in the gastrointestinal tract. A number of agents targeting the inflammatory activity of sphingosine kinases (SphK1, SphK2) and S1P receptors are currently being tested in preclinical and clinical studies[5]. Amiselimod (MT-1303), etrasimod (APD-334), ozanimod (RPC-1063), and KRP-203 (2-amino-2-1,3-propanediol hydrochloride) belong to the class of S1P modulators currently in clinical development for IBD, and they help prevent lymphocyte migration into the gut[3]. Gut T lymphocyte colonization is a critical factor in chronic gut inflammation, considering the role they play in inflammatory immune responses and immune cell interactions. Overexpression of SphK kinases and the SphK/S1P axis are associated with the regulation of inflammation in the tumor microenvironment and mediate the development of gastric and colon cancer[7]. S1P receptor modulators (FTY720, ozanimod, etrasimod) have been proposed as therapeutics for gastrointestinal cancer due to their potential anti-inflammatory effects[8]. Elucidating the different mechanisms of action of therapeutics for IBD could be an important factor in selecting the right drug for the right patient.

In this brief review, we describe the main biological mechanisms of S1P receptor modulators in IBD and how these mechanisms might influence disease progression according to current experimental and clinical data.

ROLE OF T LYMPHOCYTES AND CYTOKINES IN INFLAMED IBD TISSUE

T helper (Th) lymphocytes play a key role in modulating immune responses in the intestinal mucosa by secreting cytokines and influencing the activity and function of other immune cells[9]. In the context of immune homeostasis, the intestinal mucosa has inflammatory immune responses under control, which are regulated by a delicate balance of Th1, Th2, Th3, Th9, Th17, and regulatory T cells (Tregs)[10-12]. Naive T cells circulate through secondary lymphoid organs until they interact with antigen-presenting cells (APCs) (*e.g.*, dendritic cells, macrophages, B cells) in the gut-associated lymphoid tissue, where they encounter their cognate antigen presented by APCs. This interaction leads to the activation and proliferation of T cells, which are also imprinted into a gut homing phenotype. Imprinted T cells express specific integrins and chemokine receptors to either settle in the small intestine or migrate to the colon [13-15]. The extravasation process of T cell homing begins with the binding and rolling of T cells across

the endothelium, which is mediated by the binding of selectins and integrins on T cells to their ligands on endothelial cells. This binding slows down T cells and activates them through tissue-activated chemokines [C-X-C motif chemokine ligand 10 (CXCL10), chemokine ligand 25 (CCL25)]. Conformational changes of integrins during the interaction between T cells and endothelium lead to the arrest of activated T cells, followed by transmigration through the endothelium into the intestinal tissue (Figure 1).

The development of IBD has been linked to the synergistic effects of Th cell activity, cytokines, antimicrobial peptides, and endoplasmic reticulum (ER) stress[16-18], which initiate signaling cascades leading to the activation of key pro-inflammatory transcription factors, including nuclear factor-kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3), which further amplify and integrate signals from various stimuli[19].

In IBD, an imbalance has been observed between pro- and anti-inflammatory cytokines, which are released by the intestinal mucosa and influence the duration and intensity of inflammatory responses [20]. An accumulation of Th1 cells has been observed in IBD[21]. The proliferation and differentiation of Th1 cells is induced by interleukin 12 (IL-12)-secreting APCs[22]. Th1 cells produce interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) cytokines, which can exacerbate chronic epithelial damage of the intestinal mucosa, as they are thought to control beta-catenin signaling of intestinal epithelial cells and limit their differentiation and proliferation during intestinal inflammation[23]. IFN-γ production in colitis has been shown to induce other cells of the innate immune system to secrete inflammatory cytokines, thereby increasing chronic inflammation[24]. A specific IFN-γ+ cytotoxic CD4+ T cell subset directly promotes apoptosis of intestinal epithelial cells and intestinal enteroids in an *in vivo* colitis model[25].

In UC, both IL-10 and IL-13 cytokines are significantly increased, but the anti-inflammatory activity of IL-10 is not sufficient to reduce the activity of IL-13. The latter is restricted to the inflamed areas of the intestinal mucosa and is associated with epithelial barrier damage, cell apoptosis, decreased mucosal repair rate, and alteration of tight junctions, which negatively affects mucosal permeability[26,27].

Th17 cells are another important player in intestinal inflammation. They produce the cytokines IL-17 and IL-22, which are associated with the initiation of colitis, as they can trigger and amplify multiple inflammatory pathways. Th17 cells can also be converted to a Th1 cell phenotype in response to inflammatory cytokines (IL-12, IL-23)[28]. A disturbed Th17/Treg balance is thought to be responsible for the development of IBD. Th17 cells enhance inflammatory responses, while Tregs suppress intestinal inflammation and autoimmunity. Treg deficiency and an increase in Th17 activity have been observed in IBD[29].

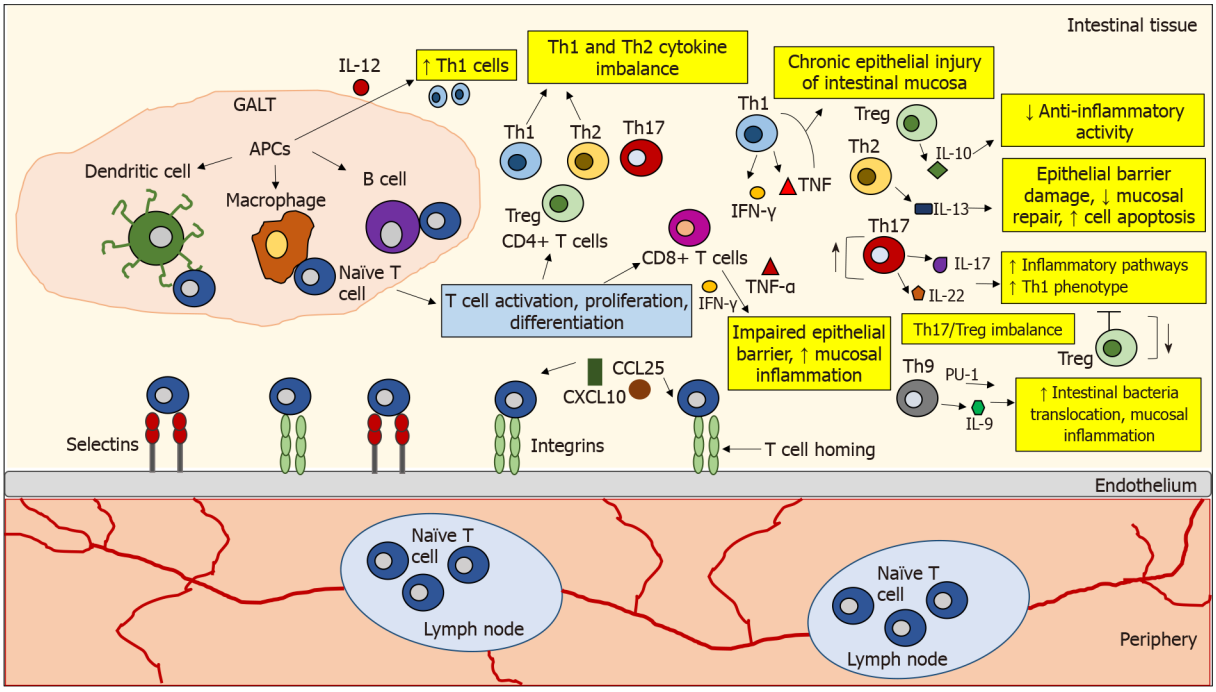
The role of Th9 cells in mucosal inflammation has also been highlighted in experimental and human UC because expression of the transcription factor PU.1, a regulator of cellular communication, and secretion of IL-9 by Th9 cells can prevent the proliferation of intestinal epithelial cells and regulate the expression of several tight junction proteins. These factors promote the translocation of certain bacterial species to the intestine, leading to subsequent immune cell activation and inflammation of the intestinal mucosa[30].

Another pro-inflammatory type of T cells recently studied in CD are γδT cells[31]. The major subtype γδ2$^+$ of γδT cells has pro-inflammatory activity after migrating into inflamed intestinal tissue, where it produces the inflammatory cytokines TNF-α and IL-17A and induces the secretion of IFN-γ from αβT cells[32].

A pathogenic mechanism mediated by CD8+ T cells in the development of IBD has also been proposed. During chronic inflammation, cytotoxic CD8+ T cells can disrupt the intestinal epithelial barrier by recognizing peptides derived from commensal bacteria on the major histocompatibility complex class I of epithelial cells and releasing the inflammatory cytokines IFN-γ and TNFα, which destroy the tight junctions of intestinal epithelial cells. When the epithelial barrier is disrupted, bacteria can invade the lamina propria and trigger an immune response mediated by innate immune cells (*e.g.*, macrophages), which induces a strong pro-inflammatory milieu that drives CD8+ T cells toward Tc1 cells or IFN+ Tregs and further exacerbates tissue damage[33]. In parallel, the decreased apoptosis of intestinal lamina propria T cells observed in IBD patients favors their pro-inflammatory effect on tissues [34] (Figure 1).

BIOLOGY OF S1P METABOLISM AND SIGNALING IN IBD

S1P is a sphingosine-derived phospholipid found in high concentration in blood and in lower concentration in other tissues. Sphingolipids contain ceramides as a structural backbone with longer, amide-linked acyl chains ranging from 14 to 36 carbon atoms in length[35]. Ceramide synthesized in the ER is hydrolyzed to sphingosine, which is then phosphorylated by SphKs (SphK1 and SphK2) to form S1P, an important regulator of inflammation[36]. S1P comprises several types of sphingolipids (S1P1-5) that act by binding to 5 G protein-coupled receptors (S1PR1-5) and by affecting key intracellular molecules that regulate gene transcription, including activation of the pro-inflammatory transcription factor NF-κB and inhibition of histone deacetylases (HDACs), which are epigenetic regulators of gene expression.



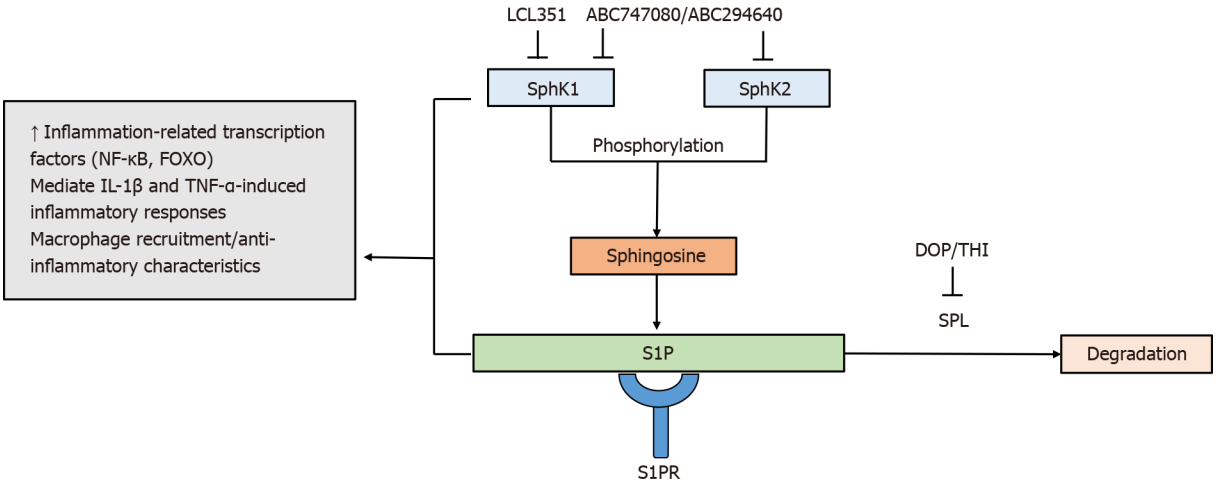
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Figure 1 The role of T lymphocytes in inflamed Inflammatory bowel disease tissue. Naive T cells circulate through secondary lymphoid organs until they interact with antigen-presenting cells (APCs) (*e.g.*, dendritic cells, macrophages, B cells) in gut-associated lymphoid tissue, where they encounter their cognate antigen presented by APCs. This interaction leads to T cell activation, proliferation, and differentiation. T cell homing begins with the binding and rolling of T cells across the endothelium, which is mediated by the binding of selectins and integrins on the T cells to their ligands expressed on the endothelial cells. This binding slows T cell activation by tissue-activated chemokines (C-X-C motif chemokine ligand 10, chemokine ligand 25). The development of inflammatory bowel disease (IBD) is associated with dysregulated activity of T helper (Th) cell subtypes. In IBD, an imbalance has been observed between pro- and anti-inflammatory cytokines released by the intestinal mucosa that influence the duration and intensity of inflammatory responses. In IBD, an accumulation of interleukin 12 (IL-12)-induced Th1 cells was observed. Th1 cells produce interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), which may enhance chronic epithelial injury of the intestinal mucosa. IL-10 secreted by regulatory T cells (Tregs) is not sufficient to counteract inflammatory activity in IBD. IL-13 secreted by Th2 cells is associated with epithelial barrier damage, decreased mucosal repair, and increased cell apoptosis. Th17 cells produce IL-17 and IL-22 cytokines that trigger multiple inflammatory pathways. Th17 cells can also be converted to a Th1 cell phenotype. A disturbed Th17/Treg balance is thought to be responsible for the development of IBD. Th9 cells can promote the translocation of intestinal bacteria and inflammation of the intestinal mucosa by expressing the transcription factor PU.1 and the cytokine IL-9. CD8+ T cells can disrupt the intestinal epithelial barrier by releasing IFN-γ and TNF-α and increase mucosal inflammation. APCs: Antigen-presenting cells; CXCL10: C-X-C motif chemokine ligand 10; CCL25: Chemokine ligand 25; GALT: Gut-associated lymphoid tissue; IL: Interleukin; IFN-γ: Interferon-gamma; TNF-α: Tumor necrosis factor-alpha; Th: T helper; Treg: T regulatory cells.

Abnormal S1P signaling has been demonstrated in preclinical colitis models, suggesting that targeting S1P production or interaction with S1PRs may alleviate colitis and reduce disease severity[37-39]. Metabolomic analysis of colon biopsies from IBD patients has revealed transcriptional and metabolic changes in sphingolipid metabolism that are thought to influence inflammation and intestinal mucosal integrity[40].

The SphK/S1PR network has been associated with the induction of inflammation-related transcription factors, including NF-κB[41] and forkhead box O[42]. The SphK/S1P axis has been shown to mediate inflammatory responses, induced by various pro-inflammatory effectors such as IL-1β and TNF-α[43,44]. Immune responses mediated by monocytes and macrophages also activate SphK/S1P signaling. Human monocytes express all five S1PRs[45], which have been shown to regulate monocyte chemotaxis and apoptosis[46]. Macrophage recruitment and manifestation of their anti-inflammatory properties have been shown to be modulated by increased activity of SphK1[47] (Figure 2). Increased expression of SphK1 has been demonstrated in IBD animal models and in human colon tissue from IBD patients, where it mediated colon damage during intestinal inflammation[48]. The SphK1/S1P/S1P1 axis was suggested to be an important signaling link between NF-κB and STAT3 transcription factors in a colitis animal model, which may have a significant impact on the relationship between chronic inflammation and colitis-associated cancer[49]. A possible role of sphingosine phosphate lyase (SPL), which degrades S1P, has been highlighted in colon carcinogenesis. SPL is expressed in differentiated enterocytes, Paneth cells, and inflammatory cells[50] and has been shown to be downregulated in colon carcinomas, resulting in increased S1P levels in neoplastic intestinal tissue[51].

S1P is a second messenger involved in the regulation of various biological processes and cellular activities, including cell survival, differentiation, migration, proliferation, immune response, trafficking of T and B cells, and cancer pathogenesis[52]. S1P also has a significant impact on the barrier function of the intestinal epithelium, as it has been shown *in vitro* to increase the concentration of E-cadherin, an



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Figure 2 Targeting sphingosine-1-phosphate metabolism by sphingosine-1-phosphate modulators. Sphingosine kinases (SphK1/SphK2) phosphorylate sphingosine to form sphingosine-1-phosphate (S1P). S1P degradation is mediated by sphingosine phosphate lyase. Components of S1P metabolism are involved in inflammatory responses. The SphK/S1P receptor network is associated with the induction of inflammation-related transcription factors, including nuclear factor-kappa B and forkhead box O. The SphK/S1P axis has been shown to mediate inflammatory responses induced by pro-inflammatory cytokines interleukin 1-beta and tumor necrosis factor-alpha. Increased activity of SphK1 modulates the recruitment of macrophages and the manifestation of their anti-inflammatory properties. S1P modulators targeting components of S1P metabolism contribute to the regulation of inflammatory immune responses. FOXO: Forkhead box O; IL: Interleukin; TNF-α: Tumor necrosis factor-alpha; NF-Kβ: Nuclear factor-kappa B; S1P: Sphingosine-1-phosphate; SPL: Sphingosine phosphate lyase.

important adherens junctions protein, thereby improving barrier integrity[53]. *In vitro* studies have also shown that S1P can improve intestinal barrier integrity by reducing TNF-α-dependent disruption[54]. S1P1 binds to S1PR1 to regulate the extravasation and migration of lymphocytes from peripheral lymphoid organs to other tissues. The controlled S1P/S1PR1-dependent mechanism is also responsible for preventing extensive trafficking of T lymphocytes into inflamed tissues after antigen-mediated T cell activation. The increased level of S1P in blood leads to the internalization of S1PR1, and the decrease in S1PR1 expression in lymph nodes and tissues allows T cells to interact with APCs[55]. The re-expression of S1PR1 on the surface of T lymphocytes after several hours causes them to migrate from the lymph node or tissue into the blood, where S1PR1 senses the increased S1P and regulates the transport of immune cells into the bloodstream[3]. Elevated S1P levels in colitis *in vivo* have been shown to activate the transcription factor STAT3 and the production of the NF-κB-regulated cytokine IL-6, initiating a signaling cascade that leads to the upregulation of S1PR1[56]. Increased levels of S1PR1 and SphK1 have been detected in the inflamed intestinal mucosa of patients with UC[57].

Small molecules acting as S1PR modulators can prevent lymphocyte invasion of inflamed tissues by the process described above. S1PR1 agonists can induce persistent lymphopenia by leading to internalization and subsequent ubiquitination and proteasome degradation of the receptor. This prevents lymphocytes from leaving the lymph node because they cannot recognize S1P levels[55,58]. S1P2 acts as a pro-inflammatory factor and, together with S1P3, mediates vascular and intestinal vasoconstriction and fibrosis[59], and S1P2-S1P3 activity has been linked to NF-κB signaling, which is involved in cytokine production, cell survival, and inflammation[60]. *In vitro* and *in vivo* experiments have shown that the S1PR2 receptor can promote proliferation of intestinal epithelial cells and increase cell permeability, possibly by regulating the expression of SphK2, HDAC1, HDAC2, and the extracellular signalregulated protein kinase 1/2 (ERK1/2) pathway. Suppression of S1PR2 in the dextran sodium sulfate-induced colitis model has been shown to ameliorate pathological damage in the colon[61]. Rodent colitis induced by deoxycholic acid (DCA) has been associated with enhanced induction of S1PR2, which together with DCA-stimulated ERK1/2 protein kinases and released lysosomal cathepsin B, mediates the activation of NLR family pyrin domain containing 3 inflammasome formation[62]. S1P/S1PRs (1-3) can induce the expression of inflammatory mediators in rat intestinal smooth muscle cells, including IL-1 and cyclooxygenase-2 (COX-2) through activation of the transcription factor early growth response protein 1 and IL-6 through activation of the transcription factor STAT3[63]. S1P4 has an immunosuppressive function by inhibiting cytokine secretion and cytokine-driven proliferation of effector T lymphocytes and promoting secretion of the anti-inflammatory cytokine IL-10[64]. Expression of S1P4R4 on dendritic cells is associated with dendritic cell migration and cytokine production[65]. Expression of S1PR4 on dendritic cells is also involved in the regulation of Th17 cells and the production of IL-27, which promotes Treg-mediated suppression of CD8+ cytotoxic T cells. Migration of neutrophils from inflamed tissues to draining lymph nodes is promoted by S1PR4[66]. S1P5 may have an impact on immune regulation, considering its expression on endothelial cells within the blood-brain barrier[52] and the link between S1P5 expression and regulation of natural killer cell number[67].

S1PRs have been highlighted as important research targets in IBD because they regulate leukocyte migration and differentiation, endothelial function, and their interaction with immune cells, contributing to the development of intestinal inflammation[68]. S1P signaling triggered by S1P binding to S1PR, has been shown to mediate and regulate pro-inflammatory responses, including the pro-inflammatory TNF-α pathway, which is currently a therapeutic target in IBD[5]. Sphingolipid-metabolizing enzymes (SphK1, SphK2) are expressed by all cells of the gastrointestinal tract as well as immune cells, and the SphK/S1P/S1PRs signaling axis mediates both normal and pathogenic inflammatory responses by influencing lymphocyte trafficking and activation of cytokine signaling. Novel agents acting as SphK inhibitors and S1PRs antagonists are being evaluated in preclinical and clinical studies for their effect in ameliorating SphK/S1P/S1PRs-mediated exacerbation of inflammation in IBD [5]. Expression of S1PR1 and SP1R4 on T cells and expression of S1PR2 on lymphatic endothelial cells (LECs) regulate the migration of T cells through LECs and into lymphatic vessels and lymph nodes. S1PR1 and S1PR4 act differently in modulating T cell motility and binding to the adhesion molecule vascular cell adhesion protein 1 (VCAM-1). Deficiency of S1PR4 has been associated with disruption of the composition of peritoneal B cell populations and decreased immunoglobulin A levels in inflammatory colitis *in vivo*[69]. S1RP2 expression is increased in vascular endothelial cells in response to microbial components and the pro-inflammatory cytokine TNF-α[70]. S1PR2 regulates the layer structure and permeability of LECs as well as the expression of adherens junction proteins *via* the ERK signaling pathway[71]. Activated S1PR3 has been shown to induce the expression of the enzyme COX-2, an inflammatory mediator, in vascular smooth muscle cells through its interaction with calcium-dependent protein kinase C and Src tyrosines[72]. Increased activation of COX-2 and high inflammatory intensity were associated with increased S1P formation in a colitis animal model[39]. The effect of SphK/S1P/S1PR signaling on linking chronic inflammation and cancer development in the gastrointestinal tract has highlighted the important role of S1PR modulators in malignancies in IBD[7]. FTY720, a SphK1 and S1PR inhibitor, was proposed as an anticancer agent in gastrointestinal cancer cells because it led to the deactivation of cancer-related downstream signaling pathways (ERK1/2, Act, c-Myc, β-catenin), which enhanced pro-apoptotic activity and tumor regression. FTY720 regulates inflammation by inducing S1PR degradation, inhibiting SphK1 activity and expression, and disrupting pro-inflammatory NF-κB/IL-6/STAT3 signaling[7]. S1PRs may be mediators of inflammatory responses in the tumor microenvironment by exerting functions on the transport and activity of innate immune system cells[8]. S1PR1 is associated with macrophage recruitment, apoptosis and anti-inflammatory responses, dendritic cell transport and inhibition of IFN-α secretion, neutrophil and eosinophil/mast cell recruitment, monocyte transport and natural killer cell egress from the lymph nodes. S1PR2 enhances antibody-mediated phagocytosis by macrophages and regulates monocyte migration. S1PR3 and S1PR4 regulate monocyte and neutrophil recruitment. S1PR3 is involved in dendritic cell-mediated maturation, promotion of Th1 response, and suppression of Tregs. S1PR5 is related to natural killer cell exit from the bone marrow and monocyte trafficking[8]. S1PR modulators tested in preclinical and clinical studies (*e.g.*, JTE013, mocravimod, amiselimod, ozanimod, etrasimod, FTY720) have shown a promising role as immunomodulators in the prevention of chronic inflammation and the treatment of inflammatory gastrointestinal cancers[8].

S1P/S1PR MODULATORS IN IBD: BIOLOGICAL MECHANISMS AND EFFECTS ON DISEASE COURSE

The development of oral small molecules targeting S1P/S1PR signaling and metabolism has shown great promise in the therapeutic area of IBD, followed by two decades of monoclonal antibodies. S1P modulators are advantageous in IBD therapy due to their low molecular weight, oral administration, low immunogenicity, rapid action, and inexpensive production. An important mechanism of action of S1P receptor modulators is that they antagonize S1P1 receptors on lymphocytes, thereby inhibiting their migration from secondary lymphoid organs to the periphery, resulting in a decrease in the number of circulating lymphocytes, including autoreactive T cells, thus causing immunomodulation[73]. Another indirect function of S1P drugs in IBD is to affect S1P metabolism by being able to inhibit the activity of components of S1P metabolism (Figure 2). Several novel selective S1P modulators are currently in development and are being evaluated for efficacy and safety profile in preclinical and clinical studies (Table 1 and Figure 3).

BIOLOGICAL FUNCTION OF S1P/S1PR MODULATORS AND PHASE OF CLINICAL DEVELOPMENT IN IBD

Amiselimod (MT-1303)

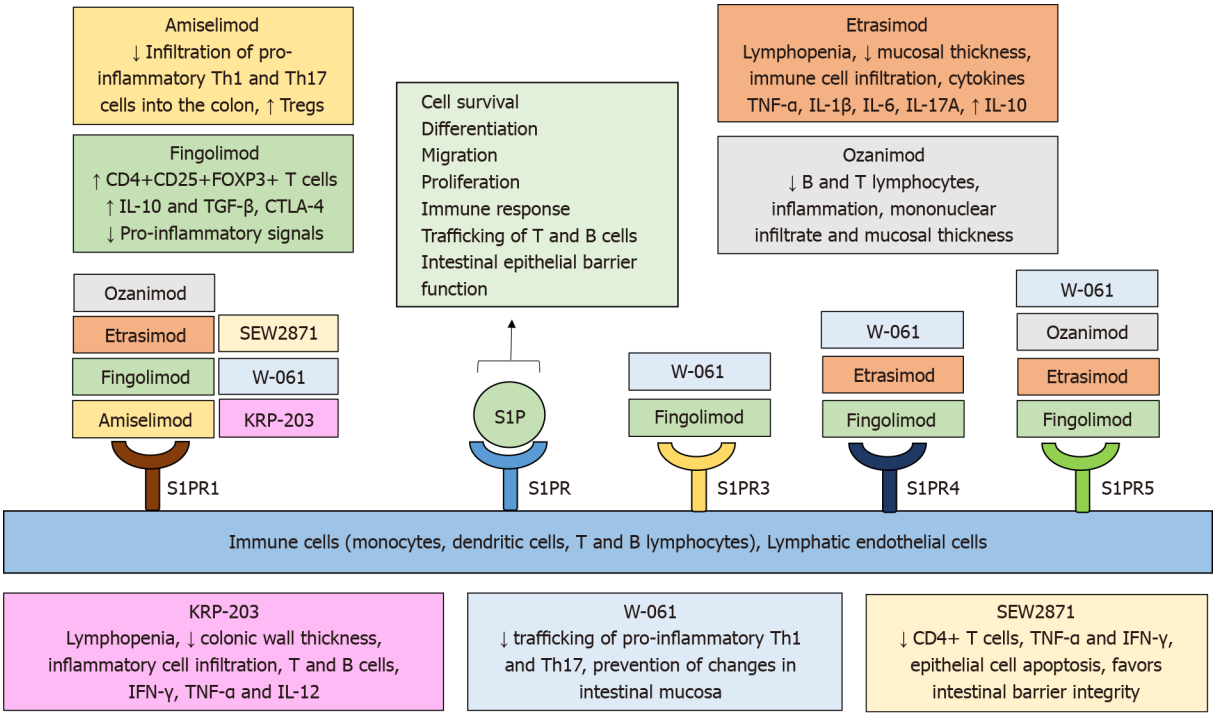
Amiselimod (MT-1303) is an oral selective modulator of the S1P1 receptor that is converted *in vivo* by

Table 1 Sphingosine-1-phosphate modulators and mechanism of action in inflammatory bowel disease

S1P modulator	Mechanism of action in IBD	Developmental phase	Ref.
Amiselimod (MT-1303)	Modulator of the S1P1 receptor→ S1P1; Internalization into lymphocytes→↓migration to the periphery; ↓Infiltration of pro-inflammatory Th1/Th17 cells into the colon; ↑Tregs in mesenteric lymph nodes	Phase IIa	[73-75]
Fingolimod (FTY720)	Specific inhibitor of S1PR1; Interferes with S1P signaling→↓lymphocyte entry into lymph nodes; ↑CD4+CD25+FOXP3+ T cells, ↑CD25, FOXP3 expression, Treg activity; ↑ Immunosuppressive cytokines IL-10 and TGF-β and CTLA-4→↑Treg-mediated immunomodulation; ↓Pro-inflammatory signaling (IL-12p70, Th1 cytokines); ↓Pro-inflammatory signals on dendritic cells→↑activity of CD4+CD25+ Tregs	Preclinical studies, not tested in humans	[76-83]
Etrasimod (APD-334)	Agonist of S1P1, partial agonist of S1P4/S1P5; Lymphopenia, ↓mucosal thickness, immune cell infiltration, expression of T-cell and monocyte markers; ↓Pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-17A, ↑anti-inflammatory IL-10	Phase II/III	NCT03996369, NCT03945188, NCT03950232, NCT04173273[84-86]
Ozanimod (RPC-1063)	Selective S1P1 and S1P5 receptor agonist; ↓T cell migration→↓peripheral lymphocytes↑S1P1 receptor internalization and degradation; ↓Circulating B and CCR7+ T lymphocytes→↓ inflammation; ↓Mononuclear cell infiltrate and mucosal thickness	Phase II/III	NCT03440385, NCT03464097, NCT03467958, NCT03440372[87-92]
KRP-203	Modulator of S1P1 receptor, partial agonist of S1PR3 receptor; Lymphopenia, ↑homing of lymphocytes to peripheral lymph nodes; ↓Infiltration of inflammatory cells in the lamina propria of the intestine; ↓CD4+ T and B220+ B cells in peripheral blood and in the lamina propria of the colon; ↓Peripheral naive and central memory CD4+ and CD8+ T cells and B cells; ↑Lymphocytes in mesenteric lymph nodes and spleen; ↓Pro-inflammatory cytokines IFN-γ, TNF-α, and IL-12 in the lamina propria of the colon	Phase II	[93-96]
LCL351	SphK1 inhibitor→↓S1P production; ↓Neutrophil infiltration into sites of inflammation, ↓ inflammatory marker TNF-α; Altered S1P levels and ↓neutrophil chemoattractants CXCL1 and CXCL2→↓leukocyte recruitment to sites of inflammation	Preclinical studies	[43,97]
ABC747080 and ABC294640	Inhibitors of SphKs→↓S1P formation, SphK activity; ↓TNF-α-induced activation of NF-Kβ; ↓ Effects of TNF-α on leukocyte recruitment and TNF-α- mediated increase in adhesion protein expression levels; ↓Pro-inflammatory cytokines (TNF-α, IL-1β, IFN-γ, IL-6) in colon tissue	Preclinical studies	[98]
DOP and THI	S1P lyase inhibitors; Peripheral lymphopenia, ↓CD4+ and CD8+ T cells; ↓Pro-inflammatory cytokines, including TNF-α, IL-6, IL-12, IFN-γ and IL-17; ↓S1PR1 expression on T lymphocytes; Depletion of late immature T cells (CD4+CD8+ double positive) and mature CD4+CD8- and CD4-CD8+ single positive cells	Preclinical studies	[99,100]
W-061	S1P receptor agonist; ↓Lymphocyte migration to the spleen and lamina propria, pro-inflammatory Th1 and Th17 cells in the lamina propria→ prevention of changes in intestinal mucosal architecture	Preclinical studies	[101]
SEW2871	S1P1 agonist; Mild inflammatory cell infiltration, ↓CD4+ T cells in the lamina propria of the colon; ↓Pro-inflammatory cytokines TNF-α and IFN-γ were significantly reduced in colonic tissues; Improved intestinal barrier function, ↑typical tight junction protein expression and distribution in the intestinal epithelium; ↓Apoptosis of intestinal epithelial cells→ restoration of colon tissue injury	Preclinical studies	[102,103]

CCR7: C-C chemokine receptor type 7; CTLA-4: Cytotoxic T-lymphocyte–associated antigen 4; CXCL: C-X-C motif chemokine ligand; FOXP3: Forkhead box P3; IBD: Inflammatory bowel disease; IFN-γ: Interferon-gamma; IL: Interleukin; NF-κB: Nuclear factor-kappa B; S1P: Sphingosine 1 phosphate; S1PR: Sphingosine 1 phosphate receptor; SphKs: Sphingosine kinases; TGF-β: Transforming growth factor-beta; Th: T helper; TNF-α: Tumor necrosis factor-alpha; Tregs: Regulatory T cells.

SphKs to its active metabolite MT-1303 phosphate (MT1303-P)[73]. The effect of MT-1303 on chronic colitis was studied in immunodeficient SCID mice induced by adoptive transfer of CD4+CD45RB^{high} T cells from BALB/c mice, an animal model for IBD. Oral administration of MT-1303 proved effective in the IBD mouse model and comparable to the effect of an anti-mTNF-α monoclonal antibody. MT-1303 significantly inhibited the infiltration of Th1 and Th17 cells into the colon by inducing the internalization of S1P1 into lymphocytes from lymph nodes and preventing their migration to the periphery. MT-1303 treatment also showed an effect on the migration of Tregs in normal mice by leading to an increased number of Tregs and an increased proportion of Tregs in mesenteric lymph nodes[74]. MT-1303 is currently enrolled and completed in a multicenter, randomized, double-blind, placebo-controlled, parallel-group, phase IIa study comparing amiselimod 0.4 mg administration to placebo over a 14-wk treatment period to evaluate safety, tolerability, and efficacy in patients with moderate-to-severe active Crohn's disease. MT-1303 treatment proved no better than placebo in eliciting a clinical response and was well tolerated, with no new safety concerns[75].



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Figure 3 Sphingosine-1-phosphate receptor modulators in preclinical and clinical studies in inflammatory bowel disease. Sphingosine-1-phosphate (S1P) receptors (S1PRs) have been highlighted as important research targets in inflammatory bowel disease (IBD). S1PRs are expressed on immune cells and lymphatic endothelial cells. Binding of S1P to S1PR regulates a variety of biological processes, including cell survival, differentiation, migration, proliferation, immune response, trafficking of T and B cells, and intestinal epithelial barrier function. Several novel, selective S1PR modulators are currently being tested in IBD and contribute to the amelioration of the inflammatory process in inflamed intestinal tissue by regulating immune cell concentration, activity, trafficking, and cytokine secretion, as well as intestinal barrier function and integrity. S1P: Sphingosine 1 phosphate; S1PR: Sphingosine 1 phosphate receptor; IBD: Inflammatory bowel disease; Th: T helper; Tregs: Regulatory T cells.

Fingolimod (FTY720)

Fingolimod (FTY720) is a specific inhibitor of S1PR1, which is a synthetic sphingosine analog (2-amino-2-2-(4-octylphenyl) ethyl-1,3-propanediol hydrochloride) of myriocin[76]. The main mechanism of action of FTY720 involves the induction of lymph node homing or sequestration of T cells. FTY720 is phosphorylated by SphK2[77] *in vivo*, and FTY720 phosphate can act as an S1P agonist that activates four of the five known G-protein-coupled S1PRs (S1P1, 3, 4, 5). S1PR1 and S1PR4 are mainly expressed on T and B lymphocytes. FTY720 interferes with S1P signaling, impeding lymphocyte entry into lymph nodes and thus delaying their subsequent return to the circulation[78]. FTY720 has not been shown to impair the activation, expansion, and differentiation of T cells to memory phenotypes, nor does it induce cell apoptosis[78-80]. FTY720 can effectively treat Th1-mediated colitis in mice by strongly affecting the activity of Tregs *in vivo*. Specifically, FTY720 administration in colitis mice was associated with increased numbers of CD4+CD25+FOXP3+ T cells and induction of CD25 and FOXP3 expression in CD4+ T cells. The immunosuppressive cytokines IL-10 and TGF-β, as well as CTLA-4, a receptor mediating the immunomodulatory function of Tregs, were also upregulated in the presence of FTY720. The therapeutic effect of FTY720 on ameliorating intestinal inflammation was associated with the downregulation of pro-inflammatory signals such as IL-12p70 and Th1 cytokines. It was suggested that FTY720-induced downregulation of pro-inflammatory signals on dendritic cells might contribute to the enhanced activity of CD4+CD25+ Tregs[81]. Although FTY720 has been approved by the Food and Drug Administration for the treatment of relapsing multiple sclerosis[82], it has not been tested in IBD patients because some adverse events (*e.g.*, elevated liver enzymes) have been noted[83].

Etrasimod (APD-334)

Etrasimod (APD-334) is a next-generation synthetic S1P receptor modulator that acts as a full agonist of human S1P1 and a partial agonist of S1P4 and S1P5[84]. Treatment with APD-334 in colitis mice resulted in dose-dependent lymphopenia, decreased mucosal thickness, and immune cell infiltration by significantly reducing the expression of T cell and monocyte markers. T cell and/or monocyte-derived proinflammatory cytokines TNF-α, IL-1β, IL-6, and IL-17A were also significantly reduced in APD-334-treated mice, and there was a dose-dependent increase in the anti-inflammatory cytokine IL-10 after APD-334 administration[85]. APD-334 has been enrolled in phase II/III clinical trials in patients with UC. In the Phase-2 OASIS study in patients with moderately-to-severely active UC with prior failure or

intolerance of conventional or biologic therapy, administration of 2 mg APD-334 resulted in significant improvement in clinical symptoms, followed by clinical remission and endoscopic improvement, with no serious life-threatening adverse events or death[86]. Moderately to severely active UC patients are currently enrolled in the ELEVATE phase III trial (NCT03996369, NCT03945188, NCT03950232). An ongoing phase II/III trial (CULTIVATE, NCT04173273) is recruiting 1265 patients with CD to evaluate the safety and efficacy of APD-334.

Ozanimod (RPC-1063)

Ozanimod (RPC-1063) is a small molecule, selective S1P1 and S1P5 receptor agonist. RPC-1063 can bind to S1P1 and S1P5 receptors, limiting the migration of T cells from peripheral lymphoid organs and reducing the number of peripheral lymphocytes[87]. The exact mechanism of action in alleviating IBD has not yet been determined. Administration of RPC-1063 in three models of autoimmune disease (experimental autoimmune encephalitis, 2,4,6-trinitrobenzenesulfonic acid colitis, and CD4+CD45RBhi T-cell adoptive transfer colitis) has shown that it induces internalization and degradation of the S1P1 receptor, leading to a reduction in circulating B and CCR7+ T lymphocytes[88], which in turn leads to a reduction in inflammation. The potential benefit of RPC-1063 for CD patients was also highlighted in a spontaneous ileitis mouse model, in which treatment with RPC-1063 resulted in a reduction in mononuclear infiltrate and mucosal thickness[89]. RPC-1063 has completed phase II/III clinical trials in patients with moderate to severe UC in which it demonstrated improved clinical, endoscopic and histologic outcomes, including reduction in rectal bleeding scores, maintenance of clinical remission, mucosal healing, histologic and durable remission. Based on the good safety profile and efficacy results, RPC-1063 was approved by regulatory authorities for the treatment of moderate to severe UC[90,91]. Histologic improvements, endoscopic remission, and a good safety profile were also reported in a phase II trial evaluating RPC-1063 in moderate-to-severe CD patients[92], and placebo-controlled phase III trials of RPC-1063 in patients with moderate-to-severe active CD are ongoing (NCT03440385, NCT03464097, NCT03467958, NCT03440372).

KRP-203 (2-amino-2-1,3-propanediol hydrochloride)

KRP-203 is a selective S1P1 modulator with a molecular structure similar to that of FTY720. Like FTY720, it induces lymphopenia by decreasing the number of lymphocytes in peripheral blood and promoting lymphocyte homing to peripheral lymph nodes[93]. KRP-203 is phosphorylated *in vivo* by SphK2. The phosphate metabolite (KRP-203 phosphate) is the active molecule that targets the S1P1 receptor and acts as a partial agonist for the human S1P3 receptor[94]. The treatment efficacy of KRP-203 in chronic colitis was investigated using a IL-10 gene-deficient (IL-$10^{-/-}$) mouse model. Mice treated with KRP-203 showed significantly reduced severity of colitis, reduced thickness of the colonic wall, reduced expansion of glandular crypts, and reduced infiltration of inflammatory cells in the lamina propria of the intestine. Administration of KRP-203 to colitis mice significantly decreased the number of CD4+T and B220+B cells in the peripheral blood and lamina propria of the colon and increased the number of lymphocytes in the mesenteric lymph nodes and spleen. After treatment with KRP-203 in colitis mice, the production of the pro-inflammatory cytokines IFN-γ, TNF-α, and IL-12 by lymphocytes in the lamina propria of the colon was significantly reduced[95]. KRP-203 was tested for its safety, tolerability, and efficacy in patients with moderately active 5-aminosalicylate-refractory UC, where it resulted in significant reductions in peripheral naive and central memory CD4+ and CD8+ T cells and B cells. KRP-203 was also found to be safe and well tolerated and resulted in clinical remission (NCT01375179)[96].

LCL351 (L-erythro-2-N-(1'-carboxamidino)-sphingosine hydrochloride)

LCL351 is a selective inhibitor of SphK1, which plays a role in S1P production. SphK1 can be activated in response to TNF-α and induce the expression of COX-2 and the production of prostaglandin E2[43]. SphK inhibitors, including LCL351, act *via* a two-way mechanism. They can either cause competitive inhibition of kinase activity or lead to proteolysis of SphK1. LCL351 showed efficacy in reducing inflammation in DSS-induced colitis in mice. LCL351 had a longer residence time in colon tissue than in blood without causing cell death. Treatment of colitis mice with LC351 blocked the infiltration of neutrophils into inflammatory sites and slightly attenuated the induction of TNF-α. The altered S1P levels mediated by LCL351, together with the reduction of neutrophil chemoattractants CXCL1 and CXCL2, may help prevent leukocyte recruitment to sites of inflammation and keep immune cells in the circulation[97].

ABC747080 (4-2-4-(4-chlorophenyl)thiazol-2-ylcarbamoyl-vinyl-2-methoxy-phenyl ester) and ABC294640 (3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide)

ABC747080 and ABC294640 are small molecules that act as selective inhibitors of SphKs and have been studied in mouse models of UC. These SphK inhibitors decreased cellular S1P formation in human endothelial cells and rat intestinal epithelial cells *in vitro* and caused a dose-dependent suppression of the activity of SphK. Treatment of fibroblasts with ABC294640 resulted in inhibition of TNF-α- induced NF-κB activation. Treatment with the SphK inhibitors ABC747080 or ABC294640 SphK *in vitro* was associated with attenuation of the effects of TNF-α on leukocyte recruitment, including TNF-α- mediated increases in adhesion protein (ICAM-1, VCAM-1) expression levels. Addition of ABC747080 or

ABC294640 to TNF-α-treated rat intestinal epithelial cells and human endothelial cell lines inhibited TNF-α-mediated induction of COX-2 activity, measured as production of PGE2. ABC294640 was also associated with decreased levels of pro-inflammatory cytokines (TNF-α, IL-1β, IFN-γ, IL-6) in colonic tissue of mice with colitis. The favorable modulation of inflammatory mediators, including S1P, NF-κB, TNF-α, VCAM-1, ICAM-1, COX-2, IL-1β, IFN-γ, and IL-6 by SphK targeting, suggests that the anti-IBD activity of SphK modulators is associated with SphK inhibition and decreased S1P synthesis[98].

DOP (4-deoxypyridoxine hydrochloride) and THI (2-acetyl-4-(tetrahydroxybutyl)imidazole) SPL inhibitors

DOP (4-deoxypyridoxine hydrochloride) and THI (2-acetyl-4-(tetrahydroxybutyl)imidazole) are small molecules that target S1P lyase (SPL), an enzyme that, together with phosphatases, tightly regulates S1P levels and keeps them low in tissues[99]. The potential anti-inflammatory effects of these SPL inhibitors on IBD were investigated in a TNF-driven mouse model of chronic ileitis with CD features. Mice treated with SPL inhibitors DOP and THI showed peripheral lymphopenia characterized by decreased numbers of CD4+ and CD8+ T cells. DOP treatment was also associated with a reduction in ileal mRNA transcripts of pro-inflammatory cytokines, including TNF, IL-6, IL-12, IFN-γ, and IL-17, resulting in attenuation of active (granulocytic) inflammation, chronic (lymphocytic/monocytic) inflammation, and overall inflammatory indices. DOP SPL inhibitor treatment resulted in downregulated S1PR1 surface expression on lymphocytes. DOP treatment was associated with promotion of thymic atrophy, depletion of late immature T cells (CD4+CD8+ double-positive) and mature CD4+CD8- and CD4-CD8+ single positive cells. It has therefore been suggested that the impairment of T cell maturation and thymic activity mediated by SPL inhibitors may significantly impair the anti-inflammatory effects of SPL inhibitors in IBD[100].

W-061

W-061 is an S1P receptor agonist that has been shown to bind all human S1P receptors except S1PR2. The therapeutic effect of W-061 on IBD was tested in a mouse model of DSS-induced colitis. Administration of W-061 to colitis mice suppressed the migration of lymphocytes to the spleen and lamina propria and induced their homing to secondary lymphoid tissues (mesenteric lymph nodes, Peyer's patches). Specifically, W-061 inhibited the migration of pro-inflammatory Th1 and Th17 cells into the lamina propria, prevented changes in intestinal mucosal architecture, and ameliorated the acute exacerbation of colitis[101].

SEW2871

SEW2871, a selective S1P1 agonist, was administered to IL10$^{-/-}$ colitis mice to investigate its function in alleviating chronic inflammation in IBD, as its protective effect on the development of colitis was demonstrated[102]. Treatment with SEW2871 resulted in mild infiltration of inflammatory cells, a reduced inflammatory score, and a decrease in CD4+ T cells in the lamina propria of the colon. The levels of pro-inflammatory cytokines TNF-α and IFN-γ were significantly reduced in the colon tissue of SEW2871-treated mice. A beneficial effect of SEW2871 on gut barrier function was also highlighted in this study. SEW2871 treatment prevented colonic permeability in IL-10$^{-/-}$ mice and promoted the typical expression and distribution of tight junction proteins in the intestinal epithelium. A possible beneficial effect of SEW2871 on the healing of colon tissue injury was observed, as SEW2871 reduced apoptosis of intestinal epithelial cells[103].

CONCLUSION

The development of IBD is mediated by dysregulated immune responses that initiate and maintain a vicious cycle of chronic inflammation. Understanding the immunopathogenesis of IBD is of great clinical importance for the development of effective therapeutics targeting these immunological pathogenic mechanisms. S1P modulators are orally administered small molecules that show promise in alleviating chronic inflammation and clinical symptoms in IBD patients. Their therapeutic efficacy is based on the fact that they regulate immune cell trafficking at sites of inflammation and are involved in immune cell interaction, cytokine production and pro-inflammatory signaling, as well as contributing to gut barrier function. The S1P receptor agonists etrasimod and ozanimod have shown favorable efficacy and safety profiles in UC and CD, patients respectively, and have significantly improved the clinical and endoscopic characteristics of IBD patients[104]. Ozanimod is being studied in ongoing phase III trials for CD (NCT03440372, NCT03440385, and NCT03464097) and UC (NCT02435992 and NCT02531126). Phase III trials (NCT03996369, NCT03945188, NCT03950232) are currently ongoing to evaluate etrasimod in patients with UC and a phase II-III trial (NCT04173273) is ongoing for etrasimod in CD. Although ozanimod has been proposed as first-line therapy in IBD, its appropriate positioning in the therapeutic algorithm for the treatment of UC treatment has not yet been defined, while its potential therapeutic activity in CD remains to be clarified[105]. KRP-203 has been shown to be safe and well tolerated in UC

patients without reaching the relevant threshold for efficacy, indicating the need for improved study design and recruitment of a larger population[96]. Determining the appropriate treatment duration of S1P modulators may pose another problem in their therapeutic use in IBD, considering that an increased risk of progressive multifocal leukoencephalopathy was observed during treatment with ozanimod and fingolimod, which was associated with a longer treatment duration in patients with multiple sclerosis[104,106,107]. The satisfactory efficacy and safety profile of S1P modulators in clinical trials, combined with their structure and advantageous production characteristics underscores their novel perspective for the treatment of IBD. Ongoing studies need to further elucidate their positioning within current treatment algorithms, their potential as combination therapies, and potential complications associated with their treatment regimens.

FOOTNOTES

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Country/Territory of origin: Greece

ORCID number: Evanthia Tourkochristou 0000-0003-1586-6854; Athanasia Mouzaki 0000-0001-5548-7002; Christos Triantos 0000-0003-3094-8209.

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MINIREVIEWS

Management of metabolic-associated fatty liver disease: The diabetology perspective

Mohammad Sadiq Jeeyavudeen, Shahanas K A Khan, Sherouk Fouda, Joseph M Pappachan

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Mohammad Sadiq Jeeyavudeen, Department of Endocrinology and Metabolism, University Hospitals of Edinburgh, Edinburgh EH4 2XU, United Kingdom

Shahanas K A Khan, Joseph M Pappachan, Department of Endocrinology and Metabolism, Lancashire Teaching Hospitals NHS Trust, Preston PR2 9HT, United Kingdom

Sherouk Fouda, School of Health and Biomedical Sciences, RMIT University, Melbourne, VIC 3046, Australia

Joseph M Pappachan, Faculty of Science, Manchester Metropolitan University, Manchester M15 6BH, United Kingdom

Joseph M Pappachan, Faculty of Biology, University of Manchester, Manchester M13 9PL, United Kingdom

Corresponding author: Joseph M Pappachan, FRCP, MD, Professor, Senior Editor, Senior Researcher, Department of Endocrinology and Metabolism, Lancashire Teaching Hospitals NHS Trust, Sharoe Green Lane, Preston PR2 9HT, United Kingdom.
drpappachan@yahoo.co.in

Abstract

The metabolic syndrome as a consequence of the obesity pandemic resulted in a substantial increase in the prevalence of metabolic-associated fatty live disease (MAFLD) and type 2 diabetes mellitus (T2DM). Because of the similarity in pathobiology shared between T2DM and MAFLD, both disorders coexist in many patients and may potentiate the disease-related outcomes with rapid progression and increased complications of the individual diseases. In fact, awareness about this coexistence and the risk of complications are often overlooked by both hepatologists and diabetologists. Management of these individual disorders in a patient should be addressed wholistically using an appropriate multidisciplinary team approach involving both the specialists and, when necessary, liaising with dieticians and surgeons. This comprehensive review is to compile the current evidence from a diabetologist's perspective on MAFLD and T2DM and to suggest optimal management strategies.

Key Words: Metabolic syndrome; Metabolic-associated fatty liver disease; Type 2 diabetes mellitus; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Diabetology perspective; Obesity

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Core Tip: The prevalence of metabolic-associated fatty live disease (MAFLD) and type 2 diabetes mellitus (T2DM) has increased exponentially as a consequence of the obesity pandemic across the globe. The pathobiology of T2DM and MAFLD are similar because both these disorders occur as a consequence of metabolic syndrome, and often coexist in many patients potentiating adverse outcomes and progression of individual diseases. However, the awareness about this coexistence is still inadequate even among hepatologists and diabetologists. A multidisciplinary team approach involving both the specialists is crucial in the optimal and wholistic management of both the disorders which is the theme of this comprehensive review.

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INTRODUCTION

The global prevalence of obesity is increasing daily because of adverse lifestyles such as overconsumption of a high-energy diet and lack of adequate physical activity. This is associated with various metabolic disorders, particularly metabolic-associated fatty liver disease (MAFLD) and type 2 diabetes mellitus (T2DM). MAFLD, previously termed as nonalcoholic fatty liver disease (NAFLD), is now identified as the most common cause of chronic liver disease in the world, affecting more than 30% of the global population[1].

Due to the similarity in pathogenesis, MAFLD is often associated with other lifestyle-related disorders such as hypertension, dyslipidemia, and most importantly T2DM, which significantly increases cardiovascular disease (CVD). MAFLD has also been found to be associated with excess risk of extrahepatic cancers, gallstones, gastro-oesophageal reflux disease, hypothyroidism, urolithiasis, chronic kidney disease (CKD), depression and worse maternal and foetal outcomes during pregnancy [2]. MAFLD is also associated with a substantial increase in cardiovascular morbidity and mortality, with a 3.5 times higher risk of heart failure and 1.93 times excess risk of all-cause CVD mortality as per recent study data in patients with T2DM[3].

In fact, the new definition for disease (MAFLD) was proposed in 2020 by replacing the old terminology NAFLD to reflect its highly remarkable link to metabolic syndrome (MetS) and the consequences such as T2DM[4]. While NAFLD was originally defined based on the imaging and/or histological evidence of steatosis in the absence of significant alcohol consumption and the exclusion of hepatitis from other etiologies, MAFLD diagnosis requires only the presence of metabolic dysfunction in persons with steatosis, and does not need excluding other etiologies of hepatitis. When MAFLD/NAFLD only involves liver steatosis without significant damage to the hepatocytes, nonalcoholic steatohepatitis (NASH) involves inflammation of liver cells with varying degrees of hepatocyte destruction, which may lead on the cirrhosis and end stage liver failure[5].

The prevalence of MAFLD in patients with T2DM varies widely in the published literature, with a recent study reporting a rate of 68% among those with obesity and T2DM[4]. The global epidemiologic data suggests that the pooled prevalence of T2DM in those with MAFLD is 22.5%, with 43.6% of those with advanced disease (NASH)[5]. Therefore, management strategies for each disease entity should definitely address the coexistence of MAFLD and T2DM, for optimal disease outcomes. In this evidence-based review, we update the management of MAFLD with due consideration of the interlink between MAFLD and diabetes to enable physicians to approach patients having better diagnostic and therapeutic perspectives.

To compile the best and most up-to-date evidence, we performed a PubMed search using the MeSH terms/key words: "metabolic-associated fatty liver disease/MAFLD", "nonalcoholic fatty liver disease/NAFLD", "type 2 diabetes mellitus/T2DM/T2D", "diabetes/diabetology/ diabetologist", "obesity" "nonalcoholic steatohepatitis/NASH", "metabolic syndrome/ MetS", "fatty liver", "steatosis", "pathophysiology", "lifestyle intervention", "exercise", "diet", "pharmacotherapy", "bariatric/metabolic surgery", "pregnancy", "children", and "elderly/ old age". The last date of search was 25th November 2022 and we used a Boolean search strategy using terms 'AND' or 'OR' where necessary to limit the search output. We limited the use of published literature in English language preferably from the most recent clinical guidelines, systematic reviews, randomised controlled trials, and high-quality review articles to procure the best available evidence on the topic of discussion while writing this narrative review paper.

MAFLD AND DIABETES: THE PATHOBIOLOGICAL INTERLINK

MetS, which encompasses central adiposity, insulin resistance, hypertension and atherogenic dyslipidemia, is the common consequence of obesity. MAFLD is considered as the hepatic manifestation of MetS, and has a bidirectional and solid relationship with other comorbidities[6]. T2DM, the predominant sequel of MetS, is therefore commonly associated with MAFLD and vice versa because of the similarity in pathogenesis. Various genetic predisposing factors, insulin resistance (IR), proatherogenic dyslipidemia [low levels of high-density lipoprotein cholesterol (HDL-C) as well as elevated concentrations of very low-density lipoproteins, triglycerides, and apolipoprotein B100], and alterations in gut microbiota resulting in intestinal dysbiosis are implicated in the development MAFLD[7].

IR, the key factor in the development of both disorders, grossly alters the hepatic glucose and lipid metabolism[7-9]. Fasting hyperglycemia as a consequence of IR and postprandial hyperglycemia from the inability of the liver to store glucose as glycogen are both implicated in the development of hepatic steatosis. IR also increases the de novo lipogenesis and triglyceride production in the liver as a result of carbohydrate-rich diet in experimental models[10]. Increased liver fat, in turn, reduces hepatic insulin clearance, insulin sensitivity and augments total body insulin resistance[11]. These factors aggravate the risk of T2DM, and in those with pre-existing T2DM, a worsening of the disease. Advanced stages of MAFLD such as NASH and cirrhosis can increase the risk of development and worsening of T2DM[5, 12-14]. Therefore, without addressing both the disease states, we cannot manage patients with MAFLD and T2DM. Figure 1 demonstrates the pathobiological interlink between T2DM and MAFLD.

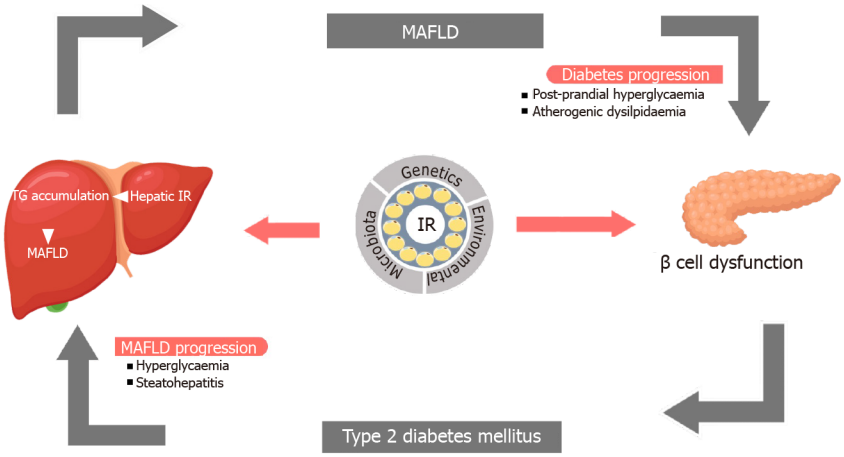
DIAGNOSTIC EVALUATION FOR MAFLD IN THE PRESENCE OF METS AND DIABETES

By definition, MAFLD is associated with the accumulation of fat in > 5% of hepatocytes on histological evaluation or using imaging evidence of > 5.6% of liver fat on magnetic resonance spectroscopy[12-14]. Various professional bodies such as the American Association for the Study of Liver Diseases (AASLD) [13], European Association for the Study of the Liver (EASL)[12], the American Association of Clinical Endocrinology (AACE)[14], and the American Diabetes Association (ADA)[15], recommend screening for MAFLD in patients with diabetes. While the EASL guideline recommends screening patients using liver function tests (LFT) and/or ultrasound scan in all cases with obesity/MetS/T2DM, the AASLD suggests screening only those with a high index of suspicion for MAFLD or NASH because of the uncertainties about the diagnosis, treatment, and the natural history of the disease. The AASLD also recommends non-invasive screening strategies like NAFLD fibrosis score, fibrosis-4 (FIB-4) score, or transient elastography (TE) for the evaluation of risk of liver fibrosis.

Based on grade B, intermediate/high strength of evidence, the latest guideline by AACE in 2022 recommends that more rigorous screening strategy should be considered for patients with T2DM using FIB-4 score, even if they have normal LFTs[14]. This guideline also advises that clinicians should consider screening for MAFLD and advanced fibrosis in those with obesity and/or features of MetS, prediabetes, and those with liver steatosis on imaging study and/or high liver transaminase levels > 6 mo as they have "high risk" for advanced fibrosis. Those with intermediate to high FIB-4 scores are considered "high risk" and should be recommended a liver stiffness measurement by TE or enhanced lifer fibrosis (ELF) test[14].

The most validated and cost-effective screening test to assess the risk of fibrosis, to identify advanced disease and liver-related outcomes in MAFLD is the vibration controlled transient elastography[14]. The most accurate imaging technique, magnetic resonance elastography, should be reserved for selected cases because of the cost implications. Alternative tests such as shear wave elastography and steatosis, activity and fibrosis (SAF) score and NAFLD activity scoring (NAS) system are also useful for screening and risk stratification. The AACE guidelines (2022) also suggests considering screening for patients with type 1 diabetes mellitus (T1DM), using FIB-4 to exclude MAFLD with clinically significant fibrosis (stages F2-F4) in presence of obesity, features of MetS, deranged LFTs with transaminase levels (> 30 U/L), or liver steatosis on imaging studies.

Because of the close association of MAFLD with diabetes and the risk of worsening of either condition among the sufferers, all patients with one of these disease entities should be evaluated for the other disease and regularly monitored using clinical and biochemical markers for each condition. Patients with either T2DM or T1DM and cardiometabolic risk factors and/or elevated transaminases (> 30 U/L) should be further risk stratified using the FIB-4 scoring, TE, and/or ELF test to exclude advanced liver disease related to MAFLD[14]. Those with persistently elevated liver transaminases and/or the presence of steatosis upon imaging evaluation and categorized as intermediate or high risk based on biochemistry and/or imaging should be referred to a hepatologist for further assessment. Liver biopsy is now considered only rarely in the diagnostic evaluation of MAFLD because of the invasiveness of the test, the high risk of complications, and the availability of high quality non-invasive diagnostic strategies in recent years for all categories of patients[14,16-18]. Sequential testing for MAFLD using blood test and non-invasive testing does reduce the number of liver biopsies required for the confirmation. In the data from the STELLAR studies, sequential testing alone had a reasonable



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Figure 1 Pathobiological link between metabolic-associated fatty live disease and type 2 diabetes mellitus. Metabolic-associated fatty live disease (MAFLD) may increase the risk for hyperglycemia and dyslipidemia and cause β-cell dysfunction leading to insulin resistance and type 2 diabetes which in turn may aggravate MAFLD progression to nonalcoholic steatohepatitis and fibrosis. IR: Insulin resistance; MAFLD: Metabolic-associated fatty live disease; TG: Triglycerides.

specificity and sensitivity with area under the curve between 0.75 to 0.80 to discriminate advanced fibrosis in MAFLD patients[19]. Our recommendations are in line with AACE/AASLD 2022 guidelines where liver biopsy is required only for intermediate to high-risk fibrosis patients or NASH diagnosis or exclude other coexisting diseases[14]. Bariatric surgery provides an opportunity to perform liver biopsy and can be potentially utilised, if the diagnosis of MAFLD is uncertain (with the other non-invasive tests) prior to the procedure, while being performed for the management of obesity.

MANAGEMENT APPROACH TO MAFLD

Lifestyle interventions

Being a metabolic disorder having significant pathogenic association with adverse lifestyles, including dietary energy imbalance and sedentary behaviors, main strategies in the management of MAFLD should be tailored around lifestyle interventions and behavioral adaptations. However, the likelihood of success with these measures is often modest because of lack of adherence and therefore, physicians are often forced to opt for pharmacotherapeutic interventions. Considering the lack of availability of many approved therapeutic agents for the treatment of MAFLD, and the well-proven additional benefits such as improvement of prediabetes/diabetes, cardiometabolic risks, and the improvement physical and emotional quality of life, the importance of lifestyle interventions cannot be underscored.

The most recent AACE guidelines (2022) reinforces the importance of lifestyle interventions as part of the standard care for patients with MAFLD and obesity, MetS, diabetes/prediabetes, hypertension, dyslipidemia, and CVD[14]. Lifestyle intervention-related weight loss of 3%-5% has shown to improve steatosis[13], with a reversal of hepatic IR[20]. The resolution of NASH in a proportion of cases with improved liver inflammation has been observed with 7%-10% weight reduction[21]. Further, fibrosis regression has been observed in those losing > 10% body weight by lifestyle changes[21,22]. However, approximately 70% of these clinical trials only achieved the target weight loss of 5% from lack of compliance and poor adherence to these weight management strategies.

Dietary interventions

Various nutritional interventions targeting a weight loss ≥ 5% has shown to be effective in the management of MAFLD[14,23,24]. Aim of all forms of nutritional interventions for MAFLD is to reduce the proportion of macronutrient content of the diet to achieve total energy deficit by restricting intake of simple carbohydrates, saturated fat, and added sugars, along with adoption of healthier eating options like a Mediterranean diet. Recent research data also suggests that intermittent fasting and time-restricted eating behavior are associated with improvement of MAFLD[25,26].

Nutritional intervention with energy restricted anti-inflammatory diets are also associated with reduction of inflammation along with better MAFLD outcomes in a recent randomized controlled trial [27]. The trial also showed significant improvements in body weight (-7.1%), visceral adiposity (-22.3%), metabolic parameters [homeostatic model assessment of IR (HOMA-IR): 15.5%; cholesterol: -5.3%; low-density lipoprotein cholesterol (LDL-C): -4.6%; and triglycerides: -12.2%], and various biomarkers of inflammation. Strict nutritional interventions for MAFLD have also been found to be associated with

improvements of CVD risk (blood pressure and QRISK2), metabolic health [fasting glucose, glycated hemoglobin (HbA1c), and insulin levels], body composition and quality of life along with improved liver-related outcomes[18,28]. However, the long-term effects and the sustainability of these nutritional interventions are still not clear in the absence of sufficiently long-term follow-up scientific research data. Although various dietary regimens are available starting from low carbohydrate/very low carbohydrate diet, ketogenic diet and Mediterranean diet, the ultimate goal for all these dietary regimens is to aim for calorie deficiency due to restriction of macronutrient (sugar and saturated fat). Of all the dietary regimens, the evidence base is strongest for the low glycaemic index Mediterranean diet which has shown to reduce the NAFLD score (median score: -4.14, 95%CI; -6.78, -1.49) when compared to the regular diet within six months duration[29]. Mediterranean diet also has cardiovascular protection property. Due to multiple health benefits with the Mediterranean diet, several professional societies recommend it as the preferred first line approach for MAFLD patients[12,14].

Exercise interventions

Physical activity is associated with improved muscle metabolism, resulting in energy dissipation and negative energy balance, especially with a calorie-restricted dietary intake. Exercise interventions have been demonstrated to significantly improve the liver transaminases regardless of the age, although more profound benefits have been observed in younger adults[30]. Along with improvements in MAFLD, various exercise programs have also been associated with improvements in metabolic parameters such as plasma levels of glucose, insulin, HbA1c, LDL-C, triglycerides, and HOMA-IR[31,32].

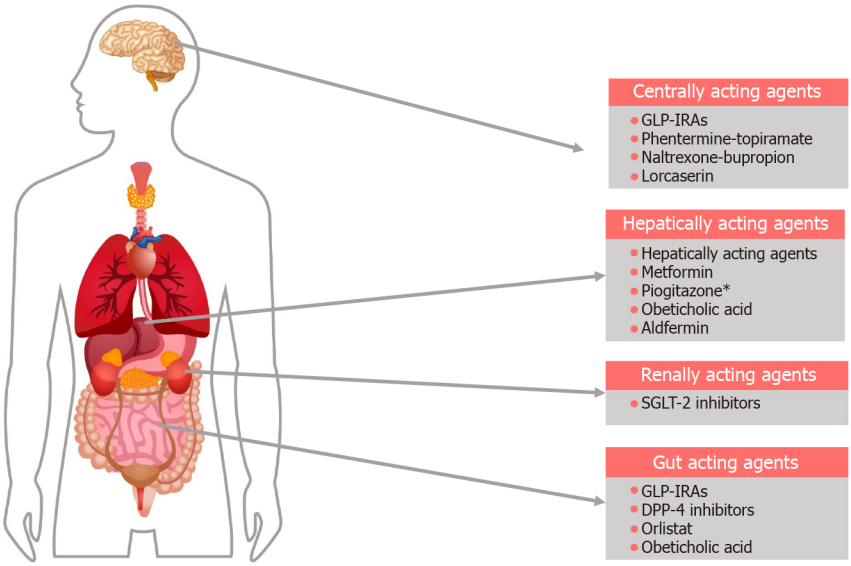
Apart from improvements in metabolic parameters, exercise interventions are also expected to improve all-cause morbidity and mortality associated with MAFLD. Both aerobic exercise and resistance training exercise result in reduction in the intrahepatic triglycerides (IHTG) content. In a randomised control trial from Thailand, a 12-wk regimen of moderate intensity aerobic exercise resulted in similar reduction of intrahepatic fat and improvement of insulin resistance in MAFLD patients when compared with resistance exercise and dietary modification[33]. A metanalysis involving six studies with 94 participants suggest that having a structured regimen results in greater reduction of IHTG and body weight[34]. Hence regular aerobic physical activity for 150-300 min/week of moderate-intensity or 75-150 min/week of vigorous-intensity coupled with some resistance training, aiming for a weight loss of > 5% in patients with MAFLD, can improve other comorbidities such as hypertension, diabetes, obstructive sleep apnea, dyslipidemia, CVD and possibly mortality[14,18,35]. Exercise interventions should ideally be coupled with nutritional changes to achieve hypocaloric energy balance to improve all these benefits. However, a recent Cochrane review concluded that there is considerable uncertainty in the evidence for benefits achieved by such interventions as the trials were of short follow-up duration of 2-24 mo[36]. Patients should be followed up for at least eight years to arrive at meaningful conclusions from these trials. Finally maintaining compliance in any chronic disorder is always challenging, different models like health behavioural model and protectional motivation theory have been proposed to improve the compliance, but all the models highlight the importance of explaining the effectiveness of the lifestyle changes to the patient and emphasising the importance of maintaining the target achieved for prevention of progression of MAFLD[37].

PHARMACOTHERAPY FOR MAFLD

Although multiple clinical trials on the pharmacotherapy for MAFLD have been completed to date, there are no drugs approved by the United States Food and Drug Administration (FDA) for the management of the disease. Most of the therapeutic agents used currently in clinical practice are based on low-moderate certainty of evidence or expert recommendations though we can expect some very promising molecules soon. Figure 2 shows the major sites of action of the drugs currently used in the management of MAFLD.

Insulin sensitizers

Pioglitazone: Pioglitazone belongs to the thiazolidinedione group of antidiabetic drugs, and acts as a peroxisome proliferator-activated receptor gamma agonist which modulates the insulin sensitivity in liver, muscles, and adipose tissues, having a marked improvement of IR[38]. Based on grade A and high strength evidence, pioglitazone is currently recommended for treatment of MAFLD when there is evidence of NASH (raised transaminases and/or suggestive non-invasive tests) in presence of T2DM[14,18,39]. Significant improvement in NASH without worsening of fibrosis was seen with the use of pioglitazone dose of 45 mg daily (relative risk: 2.64, 95%CI: 1.36; 5.12). An HbA1c reduction ranging from 1%-1.6% has been observed with neutral cardiovascular (CV) safety profile in patients with T2DM treated with pioglitazone though the use of the molecule is now less common owing to the availability of newer antidiabetic medications with better CV benefits[40]. Weight gain potential, probable risks of worsening heart failure and diabetic maculopathy from fluid retention and elevated fracture risk are the hindrances for widespread use of pioglitazone in clinical practice. The association between pioglitazone



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Figure 2 Major sites of action of drugs currently used in the treatment of metabolic-associated fatty live disease. Various medications targeting comorbidities that coexist with metabolic-associated fatty live disease are used to treat symptomatic patients. The asterisk indicates adipocyte action is not shown. GLP-1RA: Glucagon-like insulinotropic peptide-1 receptor agonist; SGLT-2: Sodium glucose cotransporter-2.

and bladder cancer is controversial. FDA has issued safety warning and advise against the use of pioglitazone in patients with active bladder cancer and to exercise caution when used in patient with previous history of bladder cancer[41]. In a meta-analysis of 26 studies, the hazard ratio for developing bladder cancer in patient with type 2 diabetes with pioglitazone exposure was 1.07 (95%CI: 0.96-1.18) and was not statistically significant with the number needed to treat for one patient to develop bladder cancer was 899 to 6380 individuals[42]. Hence detailed history about the bladder symptoms if present, warrants further investigation before the start of the medication.

Metformin: Historically, metformin has been the drug of first choice in managing patients with T2DM over the past few decades and continues to be the first-line agent even today. By reducing hepatic gluconeogenesis and increasing skeletal muscle uptake of glucose, metformin improves insulin sensitivity and ameliorates IR. Metformin therapy in patients with MAFLD has been associated with therapeutic benefits such as alanine transaminase (ALT) and aspartate transaminase (AST) reductions of -2.84 (95%CI: -11.09 to 5.28) and -2.39 (95%CI: -7.55, 2.49), respectively, along with improvements in other biological indicators like lipid abnormalities and body mass index (BMI)[43]. In patients with T2DM and MAFLD, metformin therapy and lifestyle intervention were associated with a mean weight loss of 4.3%-7.9%, leading to improvements in hepatic IR and glycemic control[44], though the drug had no efficacy in improving NASH histology[7,45]. Due to the lack of evidence for benefits, the AACE guidelines (2022) do not recommend metformin therapy for patients with NASH though the treatment for diabetes with the drug may be continued in patients with MAFLD and NASH. In the absence of sufficient clinical data on the use of the drug in T1DM and MAFLD, metformin therapy may not be recommended in such patients though the drug may be continued in those already using it.

Incretin mimetics

Incretins are gastrointestinal (GI) mucosal hormones secreted in response to the presence of nutrient stimuli in the GI lumen. Glucagon-like insulinotropic peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are the two gut hormones in this class, and both these molecules possess profound multisystemic effects on human energy balance, insulin pharmacokinetic and dynamic equilibrium, and metabolic homeostasis. Pharmacological manipulation of incretin physiology has been an area of immense global scientific research input over the past three decades that revolutionized not only diabetes therapeutics but also the knowledge regarding mechanisms of origin and evolution of a variety of metabolic and multiorgan disorders affecting human health[46,47].

The endogenously secreted GLP-1 is rapidly degraded by the gastrointestinal luminal protease enzyme dipeptidyl peptidase-4 (DPP-4) with the termination of its biological effects soon after secretion [48], with a very short elimination half-life of ≈2 min[49]. To augment the biological effects (incretin effects), and thereby improve the actions of incretin hormones, several drug molecules (incretin mimetics) belonging to GLP-1 receptor agonist (GLP-1RA) and DPP-4 inhibitor classes were subsequently developed in the early part of twenty-first century making a major paradigm shift in the management of T2DM. By augmenting the endogenous insulin production and modulating satiety,

these molecules alter the pathobiology of T2DM with weight loss potential (GLP-1RAs) or weight neutrality (DPP-4 inhibitors) making them favorite drug choices to diabetologists. Cardiovascular morbidity and mortality benefits of some of the GLP-1RAs make them more attractive choices than other antidiabetic molecules, especially because of the CVD risks associated with diabetes.

GLP-1RAs: There are several molecules of this class currently available in the market, and newer drug molecules are under development. Exenatide, Liraglutide, Lixisenatide, Dulaglutide, Semaglutide, and Albiglutide are the widely available molecules in the GLP-1RA class. The new recent agent added to this group is Tirzepatide which also possesses GIP agonist property in addition to much higher weight loss potential and improvement of HbA1c in individuals with T2DM[50]. The mean HbA1c reduction achieved with different GLP-1RAs ranges from 0.66%-2.3%[50,51], while the mean weight loss attained varies from 2.0-11.4 kg (with Tirzepatide having maximum effect)[51,52].

Along with significant improvements in body weight and glycemic control, treatment with GLP-1RAs is also associated with improvement of cardiometabolic parameters like reduction of blood pressure, positive changes in adverse lipid profile (reduction of LDL-C and triglyceride and increase in HDL-C) and reduction of proteinuria, making this antidiabetic medication class a very desirable option for physicians[53]. Many of these benefits are also highly useful in managing patients with MAFLD, especially with diabetes.

Weight loss and the improvements in diabetes control and lipid abnormalities associated with GLP-1RA therapy may be the reasons for improvement of MAFLD. Based on grade A, high strength of evidence, the 2022 AACE guidelines recommends GLP-1RA therapy for patients with T2DM and biopsy-proven NASH, encouraging clinicians to consider the drug when there is high suspicion of NASH (high transaminases and positive non-invasive tests) and to offer these molecules for cardiometabolic benefits in presence of T2DM and MAFLD[14]. Judicious use of GLP-1RAs, in particular the long acting, once weekly molecules such as Dulaglutide and Semaglutide, are expected to improve the composite outcomes such as the activity scores, transaminases and progression of disease. A recent meta-analysis showed improvements in AST [weight mean difference (WMD) = -3.29 IU/L, 95%CI: -5.98, -0.61 IU/L, P = 0.02) , ALT (WMD = -9.92 IU/L, 95%CI: -19.89, 0.05 IU/L, P = 0.05), and GGT (WMD = -12.38 IU/L, 95%CI: -15.69, -9.07 IU/L, P < 0.00001) and reduction in FIB-4 score (WMD = -0.15, 95%CI: -0.29, 0.00, P = 0.05) when compared to placebo favoring use of GLP-1RAs for treatment of MAFLD[54]. Even though there is not enough data on the benefit of Tirzepatide in managing MAFLD, the drug must be associated with more profound effects considering remarkable improvements in cardiometabolic parameters, BMI, and diabetes control with this agent. Although GLP-1RA therapy is associated with improvements in body weight, total insulin requirements and cardiometabolic parameters in patients with T1DM[55], which might potentially benefit coexisting MAFLD, recommendations cannot be made in the absence of adequate scientific proof. GI intolerance in the form of nausea, vomiting and constipation (occasionally diarrhea and abdominal pain) are common side effects of this class of medications, with a discontinuation rate of 5%-10% within a few weeks of therapy (though clinical trials report only < 5%) observed in clinical practice[56]. Higher discontinuation rates of up to 47% being reported after 12 mo and 70% by 24 mo of treatment in some reports, mostly from lack of adequate benefit and cost implications[57].

DPP-4 inhibitors: DPP-4 inhibitors are useful for the management of T2DM with the added advantage of weight neutrality when compared to many other oral hypoglycemic agents. A recent meta-analysis showed an overall HbA1c reduction of -0.53% compared to placebo with slightly better benefit in Asians compared to Whites (-0.62% *vs* -0.49%) when this drug class was used as monotherapy in T2DM[58]. However, this class of medications is not recommended by the recent AACE guidelines for MAFLD because of lack of evidence on benefits except in managing patients with coexistent T2DM. Reinforcing these recommendations, a recent meta-analysis also did not show beneficial effects on the biochemical and imaging parameters of MAFLD in patients treated with DPP4 inhibitors[54].

Sodium glucose cotransporter-2 inhibitors

Sodium glucose cotransporter-2 inhibitors (SGLT-2i) are available for the management of T2DM for nearly a decade. Noninsulin dependent glucose lowering effect with the added advantage of energy deficit induced by glycosuria make this class of drugs unique with positive cardiometabolic outcomes and modest weight loss potential. Empagliflozin, Canagliflozin, Dapagliflozin and Ertugliflozin are the common agents in this class available widely in the global market, with few other molecules available in Asia and several ones under development now. Although the AACE guidelines (2022) suggested a lack of evidence of benefit in treating steatohepatitis[14], a more recent systematic review showed remarkable benefits with the use of SGLT-2i[54]. They observed improvements in AST (WMD = -2.31 IU/L, 95%CI: -3.16, -1.47 IU/L, P < 0.00001), ALT (WMD = -5.93 IU/L, 95%CI: -7.70, -4.16 IU/L, P < 0.00001), and GGT (WMD = -6.49 IU/L, 95%CI: -11.09, -1.89 IU/L, P = 0.006) and reduction in FIB-4 score (WMD = -0.21, 95%CI: -0.40, -0.03, P = 0.02) when compared to placebo favoring the use of SGLT-2i in the management of MAFLD especially in the presence of T2DM and cardiometabolic disease.

Other antidiabetic agents

The AACE guidelines (2022) do not recommend treatment of NASH patients with antidiabetic agents such as metformin, DPP-4 inhibitors, acarbose, and insulins because of the lack of evidence for their use on improving hepatocyte necrosis and inflammation[14]. However, these agents may be continued to manage T2DM optimally in these patients. Sulphonylureas are to be used with caution as some of these medications are metabolized by the liver.

Lipid lowering drugs

Dyslipidemia, whether it is primary or secondary, is associated with a significantly higher risk of worsening of MAFLD because of metabolic dysregulation and abnormal lipid handling by the liver. Hence, the treatment of dyslipidemia is part of the routine management of MAFLD. Statins are useful in patients for the improvement of cardiometabolic outcomes, and treatment may also slow down the disease process[59,60]. Most recent data suggests that statin treatment is associated with a reduction in the steatosis grade and NAS score[61]. Therefore, statin therapy should be considered in all patients, including those with compensated cirrhosis, except when the transaminase levels are > 3 times the upper limit of normal[60]. Although Omega-3 fatty acids and fenofibrate were found to offer potential benefits in patients with hypertriglyceridemia and MAFLD in a recent small clinical trial[62], firm conclusions cannot be reached without large-scale studies. Pro-protein convertase subtilisin/kexin type 9 inhibitors are used to treat hypercholesterolemia in patients with statin intolerance or in those unable to achieve target reduction of LDL-C with tolerated doses of statins. Although these drugs are very useful for CVD protection in patients with uncontrolled dyslipidemia on conventional therapy without affecting diabetes management[63], there is not enough data on their use in patients with MAFLD to suggest recommendations for use.

Vitamin E

α-tocopherol has been found to be useful in reducing liver transaminases with improvements in histological parameters of NASH, including ballooning, inflammation, and steatohepatitis in adults[64]. PIVEN study utilised a two by two factorial study design to evaluate the efficacy of α-tocopherol, at a dosage of 800 international units daily and pioglitazone in patients with biopsy proven NASH without diabetes[64]. At the end of 96 wk, patients on vitamin E therapy showed improvement in NASH as assessed by liver biopsy when compared to placebo (43% *vs* 19%, $P = 0.001$). The long-term efficacy of vitamin E to delay the progression of NASH is yet to be determined and there are some reports of increased all-cause mortality with high dose vitamin E but this is not proven and a meta-analysis have failed to confirm this association[65]. Also, we have to bear in mind that high dose vitamin E is associated with worsening insulin resistance; therefore, the drug is not recommended in patients with T2DM and those with advanced fibrosis[14].

Anti-obesity pharmacotherapy

Orlistat: Orlistat is a pancreatic and gastric lipase inhibitor that reduces dietary fat absorption and consequently causes energy malabsorption. The efficacy and side effects depend on the dietary fat content, and the discontinuation rate is high because of steatorrhea in those consuming high-fat diets. Conflicting results were observed in the review of clinical trials examining the efficacy of orlistat in a recent meta-analysis which showed improvements in transaminases and steatosis (on ultrasonographic imaging) in a few trials where drug use resulted in weight loss, between 5%-10%, with improvements in insulin resistance also[65]. The common drug dose is 120 mg three times daily, and steatorrhea may result in discontinuation in up to 25% of patients.

A meta-analysis of 330 patients with MAFLD treatment with orlistat showed marked improvements in the BMI (mean difference = -1.97; $P = 0.02$), reduction of ALT, and improvement of insulin resistance but with no significant changes to the liver fibrosis score[66]. A randomized, placebo-controlled clinical trial from Israel noted a higher reversal rate of fatty liver (24% *vs* 17%) in orlistat-treated group for 24 wk compared to the control group[67]. Similarly, orlistat treatment for 24 wk significantly reduced liver fat content as compared to the routine treatment. In an open label parallel group study of 170 patient who were either enrolled into orlistat or conventional care in 1:1.5 proportion, showed that the level of fat content decreased to a greater extent in the orlistat treated patients compared to the conventional care, which was [-5.45% *vs* -1.96%, where < 0.001 [intention to treat (ITT) analysis] and -6.66% *vs* -2.68%, $P < 0.001$ [per-protocol (PP) analysis][68]. Higher rates of improvement in steatosis grades were seen in the orlistat treatment group [45.6% *vs* 22.5% (ITT analysis), 57.4% *vs* 30.3% (PP analysis), both $P < 0.001$] [68]. In the same study, multivariate logistic regression analysis showed that the benefit of orlistat treatment was an independent source of improvement of steatosis. In experimental animal models, orlistat has shown protective and therapeutic effects against high-fat diet-induced MAFLD *via* modulation of signaling pathways involved in improved metabolism, especially the Nrf2 signaling pathway[69]. In experimental studies, orlistat treatment in high fat diet-fed rats up-regulated several antioxidant enzymes, down-regulated cell death and inflammation and apoptosis in the testis and thus, ameliorated testicular dysfunction[70]. Overall, these data suggest that orlistat could influence the metabolism of patients, and this may offer therapeutic benefits against MAFLD.

Lesser studied anti-obesity medications: The less studied anti-obesity medications in MAFLD are phentermine-topiramate, naltrexone-bupropion and lorcaserin. These agents have predominant central action on pathways to control appetite and craving for food. Although weight loss benefit with these agents is similar or, in some cases, more than orlistat but these agents are fraught with cardiovascular complication. Hence, these agents are less preferred compared to the recent GLP-1RAs which have a better cardiovascular safety profile. In a post-hoc analysis, extended-release naltrexone-bupropion combination promoted improvement in FIB-4 score with no statistical change in the ALT when treated for six months[71]. In another study, lorcaserin treatment for six months improved fatty liver index and reduced energy intake without affecting lean mass[72]. More studies are needed specifically in MAFLD patients to clarify these agents' efficacy across various stages of MAFLD.

Other drugs used in the management of MAFLD

Obeticholic acid: Obeticholic acid (OCA), or 6α-ethyl-chenodeoxycholic acid, is a semisynthetic chemical compound that mimics bile acid (BA), chenodeoxycholic acid (CDCA) and is the first farnesoid X receptor (FXR) agonist to be approved by the FDA and European medicine agency (EMA) for primary biliary cholangitis also known as "Ocaliva"[73]. BA binds through G-protein-coupled BA receptor (TGR5/Gpbar-1) and activates the nuclear receptor (FXR), which regulates lipid and glucose metabolism. Hence, FXRs showcase an ideal target for managing MAFLD by modulating the homeostasis of cholesterol, triglyceride, glucose, energy, and BA synthesis[74]. OCA binds FXR with 100-fold affinity compared to its natural ligand CDCA and proves to be a practical analog of BA[75]. A phase 2 study on 64 humans showed that OCA administration (dose of 25 or 50 mg) for 6 wk increased insulin sensitivity, and reduced liver inflammation and fibrosis biomarkers in patients with MAFLD and T2DM[76]. In this double-blind, placebo-controlled, proof-of-concept study, patients were treated with either placebo (23 people), 25 mg OCA (20 people), or 50 mg OCA (21 people) once daily for 6 wk. Insulin sensitivity was measured by a 2-stage hyperinsulinemic-euglycemic insulin clamp before and after the 6-wk treatment period along with liver enzymes, fibroblast growth factor 19, lipid analytes, 7α-hydroxy-4-cholesten-3-one (a BA precursor), endogenous BAs, and markers of liver fibrosis. The insulin sensitivity increased by 28.0% and 20.1% from baseline in the group (20 people) treated with 25 mg OCA (P = 0.019) and in the group (21 people) treated with 50 mg OCA (P = 0.060) respectively. In the combined OCA groups, insulin sensitivity increased by 24.5% (P = 0.011) with a decrease of 5.5% in the placebo group[76]. In animal models, OCA treatment resulted in increased weight loss and improved insulin sensitivity in rabbits[77]. Similarly, OCA treatment improved the metabolic profile in MAFLD rats [Zucker (fa/fa) rats], while reducing visceral adiposity and hepatic steatosis[78]. Along similar lines, OCA treatment markedly affected the gene expression profile involved in hepatic lipotoxicity, including fatty acid synthesis, lipogenesis and gluconeogenesis genes[78]. In the FLINT trial, which involved 283 patients randomly assigned to receive OCA 25 mg daily or placebo for 72 wk showed 35% of patients treated with OCA showed marked reduction in fibrosis compared to 19% of patients treated with placebo[79]. These findings indicate that OCA would be a potential treatment option for patients with MAFLD, considering that OCA treatment improves the metabolic profile and reduces fatty liver and fibrosis. It should be noted that OCA is effective in obesity and type 2 diabetes patients, but the long-term safety data and also head-to-head comparison with other newer antidiabetic agents are still awaited.

Aldafermin: Aldafermin, also known as M70 or NGM282, is an analog of fibroblast growth factor 19 (FGF19), a human gut hormone secreted from the ileum in response to activation of FXR. This hormone is essential for bile acids, carbohydrates, and energy metabolism[80,81]. Elevations of primary and secondary bile acid in circulation are linked with primary liver disease[82]. It has been reported that FGF19, upon FXR activation, acts on the FGFR1c-KLB and FGFR4-KLB receptor complex on hepatocytes to block bile acids synthesis. Aldafermin, the FGF19 analog, acts similar way on this receptor complex. Upon activation, FGFR1c-KLB receptor suppresses the *CYP7A1* expression. This gene encodes an enzyme, cholesterol 7a-hydroxylase, which is the first and a rate-limiting enzyme for the de novo synthesis of bile acids. In the phase 2 study of 176 patients with NASH and fibrosis (biopsy-confirmed) and increased fat in the liver (> 8% by magnetic resonance imaging-proton density fat fraction), it has been shown to have a significant reduction in the serum bile acid level[83]. These patients were administered with aldafermin 0.3 mg (23 patients), 1 mg (49 patients), 3 mg (49 patients), 6 mg (28 patients) or placebo (27 patients) for 12 wk. Similarly, 62 patients with PSC and > 1.5× upper limit of normally increased alkaline phosphatase were administered with 1 mg (21 patients), 3 mg (21 patients) aldafermin, or placebo (20 patients) for 12 wk. Serum bile acid profile and neoepitope-specific N-terminal pro-peptide of type III collagen or Pro-C3 (a direct measure of fibrogenesis) were measured for metabolic and cholestatic liver diseases. The results suggested that treatment with aldafermin caused dose-dependent reductions in serum bile acids, including deoxycholic acid, lithocholic acid, glycodeoxycholic acid, glycochenodeoxycholic acid, and glycocholic acid in patients with metabolic and cholestatic liver diseases[84]. In addition, the treatment of aldafermin resulted in the reduction of glycine-conjugated bile acids but not the taurine-conjugated bile acids. Overall, these findings suggest the possibility of using aldafermin for 24 wk as a treatment option for fatty liver disease, fibrosis regression,

and resolution of NASH and stage 2 or stage 3 fibrosis.

Silymarin: Silymarin, extracted from the medicinal plant Silybum marianum, is a complex mixture of different flavonolignan isomers. Silymarin possesses an antioxidative effect and is mainly due to an isomer silybin diastereomers that is 50% of the mixture. Silybin undergoes biotransformation, forming glucuronide derivatives[85]. The mechanism of action of this drug on MAFLD involves antioxidative, choleretic, antifibrotic, regenerative, anti-inflammatory, and immunomodulatory effects[86]. Silymarin is now known as an antioxidant for reducing lipogenesis by downregulating the fatty acid synthase, peroxisome proliferator-activated receptor γ, and acetyl-CoA carboxylase[87,88]. MAFLD-induced insulin resistance and steatosis can be reduced by Silymarin which has shown the potential to restore the insulin action pathway through insulin receptor substrate-1/PI3K/Akt[89]. In a metanalysis involving 587 patients mostly from randomized controlled trials, results showed that Silymarin significantly decreased the AST and ALT levels more than the control group[90]. In a randomized, double-blind, placebo-controlled trial in NASH patients who had NAFLD activity scores of 4 or more, an intake of 700 mg per day of Silymarin for 48 wk caused a significant reduction in liver fibrosis[91]. Collectively, these studies have demonstrated that Silymarin is a safe drug, even at high doses, well tolerated, and it improves the functions of liver enzymes, activates liver metabolism, reduces oxidative stress, total cholesterol synthesis, and endothelial dysfunction in patients with MAFLD, but long-term data on different MAFLD stages are yet to come[92].

BARIATRIC PROCEDURES AND MAFLD

Metabolic surgery

One of the effective means of obtaining durable weight loss is through surgical intervention in obese people. It aims not only for weight loss but includes improvements in incretin profiles, insulin secretion, and insulin sensitivity; hence it is a promising tool for MAFLD management. Two commonly used bariatric or metabolic surgeries are Roux-en-Y gastric bypass (RYGB) and sleeve gastrectomy (SG). The fundal portion of the stomach is surgically removed during SG, whereas RYGB entails making a small pouch; this new pouch is then connected to the small intestine. While both surgical procedures result in decreased stomach volume, decreased production of acid, and changed gut hormones, RYGB involves architectural reorganization of the gastrointestinal tract, potentially impacting bile acid reabsorption [93]. As the primary bile acids (PBA) are made and conjugated in the liver, excreted in the intestine, deconjugated and/or transformed to secondary bile acids (SBA) by the gut microbiota, and recycled through reabsorption in the terminal ileum, changes in stomach due to the RYGB leads reorganization of the microbiota leading to change in PBA and SBA composition[94]. It was shown that MAFLD patients' serum had more PBA/SBA ratio compared to healthy people[94]. According to the AACE recent guidelines, metabolic surgeries are reserved for patients with MAFLD and BMI 35 kg/m^2 especially if they have type 2 diabetes mellitus (level of recommendation grade B)[14]. It can be used as an opportunity for liver biopsy for histologic staging of liver fibrosis for further prognosis.

Endo-barrier treatment

Many bariatric surgeries come with certain complications and limitations, including anastomotic disruption leading to leaks and fistulae, stomal stenosis, dumping syndrome, *etc.* The endoscopically implanted and removably attached duodenal-jejunal sleeve bypass (DJSB) or EndoBarrier® (GI Dynamics Inc., Lexington MA) can also be used for the same purpose. It is anchored in the initial section of the duodenum, where it is connected to a 60-cm long polymer sleeve by a nitinol stent anchor. The sleeve shields the proximal upper small intestine's mucosa from food that has been swallowed. DJSB offers functional similarities with RYGB as it mimics some of the physiological effects. These include food excluded from the proximal small intestine, and pancreatic and biliary secretions mixed together after food has passed through the sleeve. Endo-barrier treatment has been shown to promote significant weight loss in a patient with obesity and type 2 diabetes who are otherwise eligible for bariatric surgery but declined due to personal choice. Over the 12-mo study period, the weight loss was 15 kg (95%CI: 0.62-29.38; $P < 0.05$) and BMI 4.9 kg/m^2 (95%CI: 1.1-8.7; $P < 0.005$), the confidence intervals are wide as expected for a small sample size of forty five patients[95]. The major limitation of the endo-barrier therapy is the attended complication like gastrointestinal intolerance, bleeding, and liver abscess. Although this can be reduced by improving the dietary compliance following the procedure and there are ongoing trials on how these complications can be effectively reduced so that endo-barrier can be a safe option for MAFLD management who fail medical therapy and decline surgery.

Bariatric endoscopy

Obese patient who undergoes bariatric surgery may need to demonstrate initial weight loss for pre-operative surgery optimization of metabolic profile for safe surgical outcome. Most of these patients can get benefit from endoscopic therapy. In a study of individuals with obesity and MAFLD, bariatric

endoscopy improved analytical and ultrasound parameters of insulin resistance, hepatic fat, and hypertriglyceridemia[96]. It can be suggested during short-term follow-up as an efficient and secure option, but the long-term benefits are yet to come as these are relatively newer modalities.

A broad approach for the management and referral system pathway for patients with MAFLD is shown in the Figure 3.

SPECIAL POPULATIONS WITH MAFLD

Pregnancy and MAFLD

Pregnancy-related liver diseases occur in a trimester-specific fashion, as there is biotransformation of almost the whole body in pregnant women throughout the pregnancy. Developments of primary liver diseases are common during pregnancy. MAFLD in pregnancy can result in the development of various health issues in the mother, such as complications of hypertension, postpartum hemorrhage, and premature birth of the baby[97]. In a multicentric prospective study from Korea, Lee *et al*[98] showed that 18.4% (112/608) women had MAFLD during their early pregnancy, and MALFD is an independent risk factor in the development of gestational diabetes mellitus (GDM; 36/608). The participants with GDM development showed higher prevalence of radiological steatosis (55.6% *vs* 16.1%; $P < 0.001$) and higher fatty liver index (40.0 *vs* 10.7; $P < 0.001$) and hepatic steatosis index (35.5 *vs* 29.0; $P < 0.001$)[98]. In utero, MAFLD exposed children have a greater risk of obesity at an early age and pediatric MAFLD development[99].

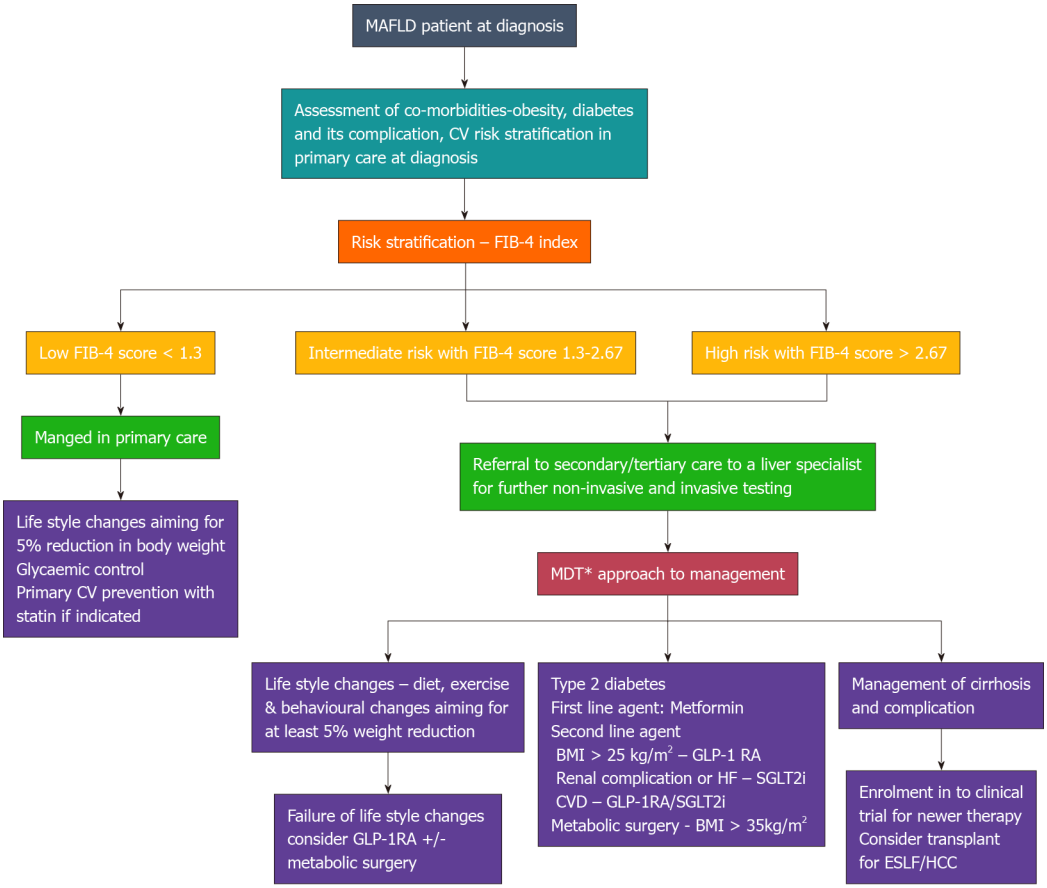
Management of MAFLD during pregnancy is a multi-model approach to control complications, which should recognize the population at high risk and control hyperglycemia, prevent excessive weight gain, and avoid the development of GDM. To our best knowledge, there is no single specific medication for the treatment of MAFLD during pregnancy. Managing weight and lifestyle during the postpartum period is mandatory for reversing the MAFLD effects and avoiding complications during the next pregnancy. Study conducted in 316 individuals (not only pregnant women), exercise without loss of weight significantly lowered intrahepatic lipid content (SMD: -0.76, 95%CI: -1.04, -0.48) and ALT concentration (SMD: -0.52, 95%CI: -0.90, -0.14), AST content (SMD: -0.68, 95%CI: -1.21, -0.15), LDL-C concentration (SMD: -0.34, 95%CI: -0.66, -0.02), and triglycerides content (SMD: -0.59, 95%CI: -1.16, -0.02)[100]. Hence, pregnant women with MAFLD should be encouraged to exercise regularly within the safe limit to improve the metabolic profile with proper guidance from the expert obstetric team.

Children and MAFLD

Due to a lack of recognition, screening, and appreciation of complications associated with MAFLD, the disease often remains undiagnosed among children. In a study of 2256 children with 715 children overweight, MAFLD was diagnosed in 23% among 715 with overweight ($P < 0.01$)[101]. As pediatric treatment options are limited due to inadequate number of clinical trials and insufficient knowledge, the overall goal is to improve the life quality of children and reduce liver morbidity and mortality. Lifestyle management is one of the best ways to manage MAFLD in children. In a study of 84 children of the age group between 3 and 18.8 years, over two years, with proper diet and physical exercise, participants who lost 5% or more of body weight had more significant improvements in the level of ALT when compared with the participants with < 5% weight loss (-35 ± 33 *vs* -20 ± 20 IU/L, respectively; $P < 0.05$)[102].

Elderly individuals and MAFLD

Between 40-50 years of age in males and 60-69 years in females, the prevalence of fatty liver is high and seems slightly low in older (> 70 years) cohorts. Hypertension, diabetes, hyperlipidemia, and obesity risk factors for MAFLD development are higher in elderly individuals. The diagnosis and management strategies are challenging in the elderly with other age-related comorbidities[103]. For the management of MAFLD, the underlying etiology must be treated parallel to other diseases including obesity, hyperlipidemia, and IR. Lifestyle modifications and pharmacological treatment are the two methods considered safe for managing MAFLD in elderly individuals. In a study with 261 elderly individuals for 52 wk, 72 (25%) achieved steatohepatitis resolution, 138 (47%) reduced NAS score, and 56 (19%) achieved fibrosis regression. During last week, 88 subjects (30%) reduced ≥ 5% of their weight. Degree of weight loss and improvements in all NASH-related histologic parameters are independently associated (odds ratios = 1.1-2.0; $P < 0.01$). A major proportion of participants with ≥ 5% loss in weight had [51 of 88 (58%)] NASH resolution and a 2-point reduction in NAS [72 of 88 (82%)] than participants with < 5% of loss in their weight ($P < 0.001$)[104]. In some elderly patients, these modifications might be incompatible. So, it is necessary to ensure that the introduced dietary instructions are not excessively aggressive and provide adequate nutrition to the elderly. Currently, no particular drug is approved for the treatment of MAFLD in the elderly. Therefore, there are no specific recommendations for drug usage in elderly individuals with MAFLD alone, but if they have concomitant T2DM, metformin, GLP-1RAs and SGLT-2i are the best available options.



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Figure 3 Management and referral system pathway for patient with metabolic associated fatty liver disease. Multidisciplinary treatment approach involving dietician, physiotherapist, psychologist, diabetologist and hepatologist. In resource constrained setting, referral to individual specialist based on the predominant effect due to metabolic-associated fatty live disease as per the pathway. GLP-1RA: Glucagon-like insulinotropic peptide-1 receptor agonist; SGLT-2i: Sodium glucose cotransporter-2 inhibitors; CVD: Cardiovascular disease; HF: Heart failure; BMI: Body mass index.

EMERGING CONCEPTS

MAFLD is a significant cause of chronic liver disease with reported increase in hepatocellular carcinoma (HCC), and a leading contributor to various systemic complications such as T2DM, CVD, and CKD. A certain degree of uncertainty is present regarding the natural history and prognosis of MAFLD. In some patients, the long evolution time for MAFLD progression from steatohepatitis to fibrosis is noted, but some progress faster from cirrhosis to hepatocellular carcinoma[105]. The exact reason for the development of HCC among MAFLD patients remains uncertain to date. When compared with chronic hepatitis C infection, the cumulative incidence of MAFLD patient developing HCC is lower (4% *vs* 2.5%) at 1 year and (30% *vs* 11%) at 5 years[106]. Moreover, patients with MAFLD has a tendency to develop HCC even without cirrhosis and the risk is five times when compared those with chronic hepatitis C infection[107]. As these patients without cirrhosis are not in the surveillance program, they tend to present with bigger tumours with reduced median survival[108]. Recent data suggest that patients who develop HCC due to MAFLD cirrhosis live longer than hepatitis C related HCC after curative treatment [109]. Liver transplantation is used either for end stage liver disease or HCC due to MAFLD. The transplant related mortality and morbidity is high compared to the HCC due to other aetiologies such as very high BMI and MAFLD related cardiovascular complications[110]. But it is interesting to note that post-transplant 5-year survival rate is not different between MAFLD and non-MALFD aetiology because the lower risk of graft failure balances the higher risk of sepsis and cardiovascular disease in MAFLD patients when compared to other aetiology[111].

More clinical trials involving different ethnicity having divergent gene pools are required to get insights into the contribution of the genetic basis of MAFLD and the genetic basis of successful treatment outcomes and failure in treatment. The effect of environmental factors should also be a primary focus of work in the next decade. The growing human population and the environmental impact have dramatically changed human life. Especially lifestyle diseases are emerging in this decade due to the impact of the environment on human life. Future studies also need to be focused on understanding this phenomenon.

Table 1 Management of metabolic-associated fatty liver disease directed against the risk factors

No	Disease/condition	Directed therapy	Supportive therapies
1	Overweight and obesity	Anti-obesity drugs, Bariatric surgery, lifestyle intervention (Calorie-restriction, dietary pattern, *etc.*)	MAFLD: Newer agents targeting on cellular inflammation and oxidative stress.
2	T2DM	Hypoglycemic agents like Metformin, GLP-1RAs, SGLT-2i, Thiazolidinediones and DPP-4 inhibitors	Fibrotic MAFLD: Potential future anti-fibrotic agents.
3	> 2 metabolic risks	Modulators of metabolism (Farnesoid X receptor agonsit, Peroxisome proliferator-activated receptor, fibroblast growth receptors, statins, aspirin)	Cirrhosis Complications: Control portal hypertension and bacterial peritonitis prophylaxis. End-stage liver diseases: Liver transplantation.

T2DM: Type 2 diabetes mellitus; GLP-1RA: Glucagon-like insulinotropic peptide-1 receptor agonist; SGLT-2i: Sodium glucose cotransporter-2 inhibitors; DPP-4: Dipeptidyl peptidase-4; MAFLD: Metabolic-associated fatty live disease.

Different pharmacological approaches are tried to reduce the risk of MAFLD. However, current strategies remain ineffective. The asymptomatic time intervals in MAFLD and NASH progression to cirrhosis and ultimately a liver failure and gaps in knowledge regarding modifiers of the disease contribute to significant challenges in the drug design for the clinical trials. The application of nanoparticles for drug delivery demonstrated potentially promising for enhancing drug bioavailability for MAFLD treatment. Various types of liver-targeting nanoparticles are exploited for MAFLD management[112].

Table 1 shows a summary of therapeutic strategy for MAFLD targeting risk factors.

CONCLUSION

MAFLD is a growing global health burden among the population with high susceptibility to obesity and insulin resistance. Notably, MAFLD is the most common cause of chronic liver disease in children and adolescents, exhibited by fatty liver disease and severe fibrosis. The most effective prevention strategy for MAFLD is lifestyle modification. Considering the prevalence and its impact, several novel therapies, including several novel therapeutic and surgical approaches, are currently under investigation. Pharmacological interventions, including treatment with antidiabetic medications, and anti-obesity drugs, can be considered for MAFLD patients but should be chosen wisely on a case-to-case basis. In addition, bariatric surgeries aimed to obtain durable weight loss are options for MAFLD patients who either fail medical management or have associated comorbidities. Novel approaches in drug delivery would be ideal for managing MAFLD in the future. We need to improve the knowledge of the personalized treatment options with targeted drugs, which would be effective in that subset of patients who fail or have difficulty adhering to the routine management pathways.

FOOTNOTES

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Country/Territory of origin: United Kingdom

ORCID number: Mohammad Sadiq Jeeyavudeen 0000-0002-1461-0534; Sherouk Fouda 0000-0001-8227-4571; Joseph M Pappachan 0000-0003-0886-5255.

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MINIREVIEWS

Role of gut microbiota in the pathogenesis and therapeutics of minimal hepatic encephalopathy *via* the gut-liver-brain axis

Ming Luo, Rui-Juan Xin, Fang-Rui Hu, Li Yao, Sheng-Juan Hu, Fei-Hu Bai

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Ming Luo, Rui-Juan Xin, Fang-Rui Hu, Li Yao, Sheng-Juan Hu, Fei-Hu Bai, Department of Gastroenterology, People's Hospital of Ningxia Hui Autonomous Region, Yinchuan 750021, Ningxia Hui Autonomous Region, China

Corresponding author: Fei-Hu Bai, MD, Chief Physician, Professor, Department of Gastroenterology, People's Hospital of Ningxia Hui Autonomous Region, No. 301 Zhengyuan North Street, Jinfeng District, Yinchuan 750021, Ningxia Hui Autonomous Region, China. baifeihu@hotmail.com

Abstract

Minimal hepatic encephalopathy (MHE) is a frequent neurological and psychiatric complication of liver cirrhosis. The precise pathogenesis of MHE is complicated and has yet to be fully elucidated. Studies in cirrhotic patients and experimental animals with MHE have indicated that gut microbiota dysbiosis induces systemic inflammation, hyperammonemia, and endotoxemia, subsequently leading to neuroinflammation in the brain *via* the gut-liver-brain axis. Related mechanisms initiated by gut microbiota dysbiosis have significant roles in MHE pathogenesis. The currently available therapeutic strategies for MHE in clinical practice, including lactulose, rifaximin, probiotics, synbiotics, and fecal microbiota transplantation, exert their effects mainly by modulating gut microbiota dysbiosis. Microbiome therapies for MHE have shown promised efficacy and safety; however, several controversies and challenges regarding their clinical use deserve to be intensively discussed. We have summarized the latest research findings concerning the roles of gut microbiota dysbiosis in the pathogenesis of MHE *via* the gut-liver-brain axis as well as the potential mechanisms by which microbiome therapies regulate gut microbiota dysbiosis in MHE patients.

Key Words: Gut microbiota; Minimal hepatic encephalopathy; Gut-liver-brain axis; Pathogenesis; Therapeutics

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Core Tip: Minimal hepatic encephalopathy (MHE) is a common neuropsychiatric complication of liver cirrhosis. Gut microbiota dysbiosis has an essential role in the pathogenesis of MHE *via* the gut-liver-brain axis. Current therapeutic strategies for MHE are based on the modulation of gut microbiota dysbiosis. This review presents the recent evidence on the roles of gut microbiota dysbiosis in the pathogenesis and treatment of MHE *via* the gut-liver-brain axis.

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INTRODUCTION

Hepatic encephalopathy (HE) is a central nervous system complication of chronic liver disease or portal systemic shunting that is characterized by a broad range of neuropsychiatric symptoms[1]. Depending on the severity of clinical manifestations, HE can be classified as overt or covert, such as minimal HE (MHE) and West Haven grade I HE[1]. Overt HE (OHE) exhibits obvious neurological and psychiatric manifestations, such as flapping tremors, drowsiness, and sometimes coma[2]. In contrast, MHE presents with slight cognitive deficits in the executive function, including psychomotor speed, response inhibition, and working memory, with no clinical evidence of OHE[3]. MHE is diagnosed using neurophysiological and psychometric tests, and its prevalence range from approximately 30% to 70% in different populations with liver cirrhosis[1,3,4]. MHE compromises daily functions, affects the health-related quality of life, and increases the risk of progression to OHE[5,6].

The exact pathogenesis of MHE is complex and not completely understood. Furthermore, the pathophysiological basis of MHE is multifactorial, with ammonia, inflammation, and endotoxins considered causative factors[7]. Recently, gut microbiota dysbiosis has been demonstrated to be associated with hyperammonemia, systemic inflammation, neuroinflammation, and endotoxemia in cirrhotic patients and experimental animals with MHE[8-11]. With decompensated liver cirrhosis and hepatic dysfunction, dysbiotic gut microbiota and its metabolites cross the impaired intestinal barrier and induce hyperammonemia, systemic inflammation, and endotoxemia, which influence the permeability of the blood-brain barrier (BBB), resulting in neuroinflammation and low-grade edema in the cerebrum and contributing to central nervous system dysfunction[7]. It has been increasingly recognized that gut microbiota dysbiosis is the predominant factor accounting for MHE pathogenesis *via* the gut-liver-brain axis[12,13]. Furthermore, an increasing number of clinical studies have shown that currently available therapies for MHE patients, including lactulose, probiotics, synbiotics, rifaximin, and fecal microbiota transplantation (FMT), improve cognitive dysfunction through the modulation of gut microbiota dysbiosis[14-17].

Although several published reviews have elucidated the involvement of gut microbiota dysbiosis in the pathogenesis and treatment of HE, no specific review has focused on this involvement in MHE[13,18-20]. Therefore, this review aimed to comprehensively elucidate the roles of gut microbiota in MHE pathogenesis *via* the gut-liver-brain axis and systematically analyze the underlying mechanisms linked with microbiome therapies to modulate gut microbiota dysbiosis in cirrhotic patients with MHE.

GUT MICROBIOTA DYSBIOSIS IN MHE

Small intestinal bacterial overgrowth (SIBO) is a pathological dysregulation of gut microbiota, characterized by excessive bacteria and/or abnormal bacterial composition in the small intestine. Approximately 48% to 73% of cirrhotic patients have SIBO[21]. Intestinal immune dysfunction, intestinal dysmotility, and decreased bile acid synthesis are implicated in the pathogenesis of SIBO[22]. SIBO is closely associated with the severity of advanced liver cirrhosis, and it has been validated as a significant risk factor for MHE[23,24]. In MHE patients, the gut microbiota dysbiosis resulting from the SIBO has been characterized by lower bacterial diversity, decreased autochthonous beneficial bacteria, and increased pathogenic Gram-negative bacteria[8,14,25]. The gut microbiota dysbiosis in cirrhotic patients with MHE is summarized in Table 1. Notably, Bajaj *et al*[26] found that MHE patients have higher abundances of *Enterococcus* and *Veillonella* and a lower abundance of *Roseburia* in the gut mucosal microbiota; these signatures are significantly different from those of the fecal microbiota. It is hypothesized that the adherence and overgrowth of pathogenic bacteria in the gut mucosal microbiota, rather than the fecal microbiota, might be implicated in the pathogenesis of bacterial translocation.

Table 1 Clinical studies of gut microbiota dysbiosis in cirrhotic patients with minimal hepatic encephalopathy

Ref.	Nationality	Number of patients	Etiology of cirrhosis	MHE diagnosis	Sample	Method	Microbiota alteration
Zhang *et al*[8]	China	51	AIH, HBV, PBC, alcohol	NCT-A DST	Stool	16S rRNA pyrosequencing	Enriched *Streptococcus salivarius*
Wang *et al*[14]	China	98	HBV, HCV, others	NCT-A DST	Stool	16S rRNA sequencing	Enriched *Proteobacteria*, especially *Pasteurellaceae Haemophilus* and *Alcaligenaceae Parasutterella*
Luo *et al* [29]	China	143	HBV	PHES	Stool	16S rRNA sequencing	Enriched *Streptococcus salivarius* and *Veillonella*
Liu *et al* [76]	China	55	HBV, HCV, alcohol, others	NCT-A BAEP	Stool	Stool bacterial culture	Overgrowth of *E. coli* and *Staphylococcus* spp.
Bajaj *et al* [27]	United States	97	HCV, alcohol, NASH, others	ICT PHES	Stool	Shotgun metagenomic sequencing	*Alistipes ihumii*, *Prevotella copri*, and *Eubacterium* spp. were higher, while *Enterococcus* spp. were uniquely lower in MHE diagnosed by ICT
Bajaj *et al* [28]	United States	247	HCV, alcohol, NASH, others	PHES ICT Stroop	Stool	Multi-tagged sequencing	Enriched *Lactobacillaceae*

AIH: Autoimmune hepatitis; HBV: Hepatitis B virus; HCV: Hepatitis C virus; PBC: Primary biliary cirrhosis; NASH: Non-alcoholic steatohepatitis; NCT-A: Number connection test-A; DST: Digit symbol test; ICT: Inhibitory control test; PHES: Psychometric hepatic encephalopathy score; MHE: Minimal hepatic encephalopathy.

Because of the lack of uniform criteria for diagnosing MHE, the results of the currently available diagnostic methods for MHE are inconsistent in clinical practice. Specific signatures of fecal microbiota correspond to unique cognitive impairments determined by different diagnostic methods for MHE, including the psychometric hepatic encephalopathy score, inhibitory control test, and EncephalApp Stroop test (Table 1). For example, the abundances of *Enterococcus* and *Streptococcus* were higher in cirrhotic patients with MHE diagnosed by the psychometric hepatic encephalopathy score only; however, the abundances of *Prevotella copri*, *Eggerthela*, and *Alistipes* spp. were higher in those with MHE diagnosed by the inhibitory control test only[27]. Of note, the *Lactobacillaceae* abundance was also higher in fecal samples of MHE patients, regardless of MHE testing; therefore, this might be able to be used as a substitution for MHE testing[28].

Gut microbiota signatures of MHE patients vary depending on the etiology of liver cirrhosis. In a Chinese cohort with cirrhosis, the abundances of *Streptococcaceae* and *Veillonellaceae* were overrepresented in cirrhotic patients, and MHE patients had a higher abundance of *Streptococcus salivarius*[8]. Moreover, *Streptococcus salivarius* was also enriched in the gut microbiome of patients with MHE due to hepatitis B-associated liver cirrhosis, especially in those with sleep disturbances[29]. In contrast, in a cohort with cirrhosis in the United States, the fecal *Lactobacillaceae* abundance was higher in MHE patients; however, the abundance of fecal *Lachnospiraceae* genera, such as *Clostridium XIVb* and *Ruminococcus*, was correlated with better cognitive function independent of clinical variables[28]. Additionally, another study in the United States revealed that a higher *Veillonellaceae* abundance was found in the fecal microbiota of MHE patients, and that *Porphyromonadaceae* and *Alcaligeneceae* were positively associated with cognitive dysfunction in MHE[30]. The altered gut microbiota in Chinese MHE patients differs from that of MHE patients in the United States because the primary etiology of liver cirrhosis in the Chinese population is hepatitis B; however, in the United States, hepatitis C and excessive alcohol consumption are the predominant etiologies[31,32].

GUT-LIVER-BRAIN AXIS IN MHE

Bacterial translocation

In healthy individuals, the characteristic structure and immune system of the intestinal mucosa can prevent bacteria and their byproducts from entering the systemic circulation. In patients with liver cirrhosis, the SIBO decreases the synthesis of secondary bile acids by inhibiting the activation of Farnesoid X receptor and Takeda G protein-coupled receptor, which reduces intestinal immunoglobulin A levels and further compromises the immune function of the intestinal mucosa[33,34]. Moreover, the SIBO induces decreased synthesis of antimicrobial peptide and activates mucosal immune responses, resulting in intestinal inflammation and impaired intestinal epithelium integrity[35]. Furthermore, the SIBO increases the permeability of epithelial intercellular junctions *via* the down-regulation of tight junction protein expressions[36]. These potential mechanisms induce a “leaky gut” which facilitates the

transfer of pathogenic bacteria and their metabolites from the intestinal tract to the circulatory system, resulting in systemic inflammation (Figure 1).

Systemic inflammation

Translocated bacteria and their products, such as pathogen-associated molecular patterns, are transported to the liver through the portal vein. At the molecular level, pathogen-associated molecular patterns are recognized by Toll-like receptors and cytoplasmic nucleotide-binding oligomerization domain-like receptors, and primarily stimulate hepatic Kupffer cells through the activation of MyD88-dependent and NF-κB signaling pathways[37,38]. Innate immune responses in the liver are triggered, resulting in liver damage, with the release of damage-associated molecular patterns and the production of proinflammatory cytokines and chemokines (Figure 1)[37,38]. MHE patients present the systemic proinflammatory environment reflected by increased circulatory levels of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukins (ILs), and interferon, and chemokines such as CCL20, CXCL13 and CX3CL1[39].

In experimental mice with MHE, higher abundances of *Staphylococcaceae*, *Enterobacteriaceae*, and *Lactobacillaceae* in the large intestine and of *Staphylococcaceae*, *Streptococcaceae*, and *Enterobacteriaceae* in the small intestine were associated with systemic inflammation along with higher circulating concentrations of TNF-α and IL-1β[10]. Similarly, the abundances of *Enterobacteriaceae*, *Fusobacteriaceae*, and *Veillonellaceae* were positively associated with higher serum concentrations of IL-2, IL-13, and IL-23 in cirrhotic patients with MHE, and these increased cytokines were significantly correlated with MHE severity[30]. Furthermore, the association between MHE severity and increased proinflammatory cytokines has been demonstrated to be independent of the severity of liver cirrhosis and ammonia levels, suggesting that systemic inflammation with its proinflammatory cytokines is potentially implicated in the development of MHE[40].

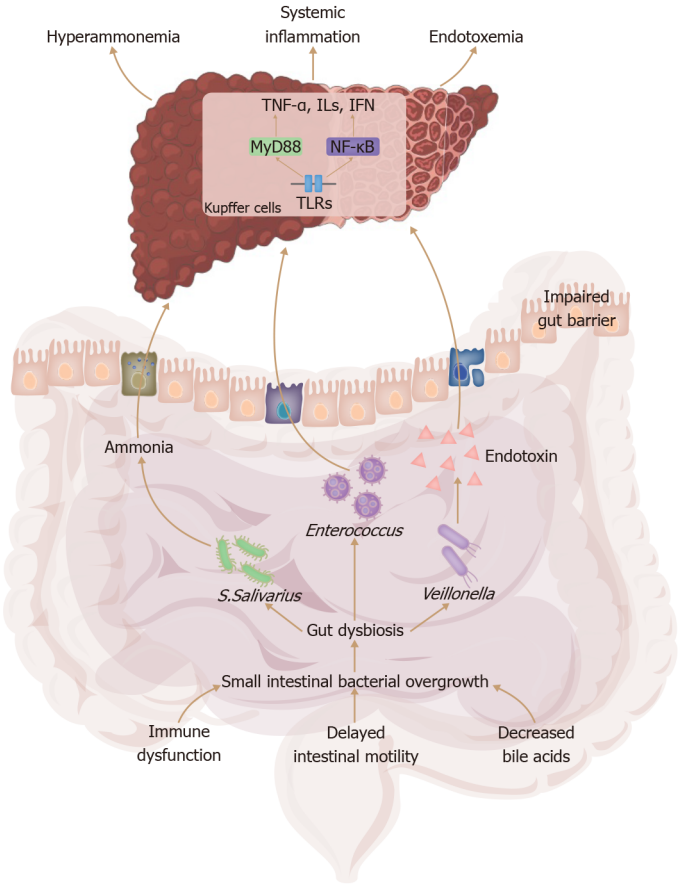
Blood-brain barrier permeability

The blood-brain barrier is composed of capillary endothelial cells surrounded by capillary basement membrane and astrocytic perivascular endfeet. The BBB separates the systemic circulation and brain, prevents the entry of potentially harmful substances into the brain, and maintains the homeostasis of the brain microenvironment. Circulating proinflammatory cytokines cannot directly cross the BBB and exert their effects on the brain. However, these cytokines, including TNF-α, ILs, and interferon, down-regulate the expression of endothelial tight junction proteins, compromise cerebrovascular endothelial cells, activate astrocytes to an inflammatory reactive state, and alter BBB receptor expression and transport pathways, which consequently impair BBB integrity and further increase BBB permeability (Figure 2)[41,42]. Through the aforementioned mechanisms, the proinflammatory signaling, which is initiated by systemic inflammation, crosses the damaged BBB and underlies the neuroinflammatory response that develops in the cerebrum.

Neuroinflammation

Neuroinflammation refers to a series of inflammatory response processes characterized by microglial activation and proinflammatory cytokine production in the cerebrum[43]. The proinflammatory signaling, originating from systemic inflammation and crossing the BBB, induces microglial activation, stimulates Toll-like receptors, and activates NF-κB and myeloid protein-dependent pathways to produce proinflammatory mediators in the cerebrum[41]. Neuroinflammation interferes with neurotransmission, affects neuronal function, and induces low-grade cerebral edema in concert with hyperammonemia (Figure 2)[43]. Balzano *et al*[44] found that MHE rats experienced not only increased serum levels of prostaglandin E2, IL-6, and IL-17 but also microglial activation with increased mRNA expression of TNF-α and IL-1β in the hippocampus, which indicated the existence of both systemic inflammation and neuroinflammation in MHE. Additionally, *Enterobacteriaceae* in the cecum and *Staphylococcaceae* in the small intestine are linked to serum proinflammatory cytokines and neuroinflammation in cirrhotic mice[10]. Moreover, germ-free mice colonized with feces from MHE patients containing high abundances of *Enterobacteriaceae*, *Staphylococcaceae*, and *Streptococcaceae* had remarkable microglial activation and neuroinflammation[9]. Therefore, neuroinflammation is closely associated with gut microbiota dysbiosis in experimental animal models of MHE.

In contrast, the neuroinflammation in MHE patients has not been extensively studied. Postmortem examination of cerebral specimens from MHE patients showed that mRNA expressions of TNF-α, IL-1β, and IL-6 remained unchanged in the cerebral cortex, although genes related to microglial activation were upregulated[45,46]. Current evidence of the involvement of gut microbiota dysbiosis in the pathogenesis of neuroinflammation has been derived from experimental animal models of MHE; however, related studies of MHE patients are lacking. Magnetic resonance imaging (MRI) has been successfully used to quantify the manganese deposition in the brain and noradrenaline in MHE rats[47,48]. It is presumed that cerebral MRI examinations of MHE patients could facilitate further research concerning the involvement of gut microbiota dysbiosis in the pathogenesis of neuroinflammation.



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Figure 1 On the background of liver cirrhosis with hepatic dysfunction, dysbiotic gut microbiota and its byproducts including ammonia and endotoxin cross the impaired intestinal barrier, stimulate innate immune responses in the liver, and lead to systemic inflammation, hyperammonemia, and endotoxemia. TNF-α: Tumor necrosis factor-alpha; ILs: Interleukins; IFN: Interferon.

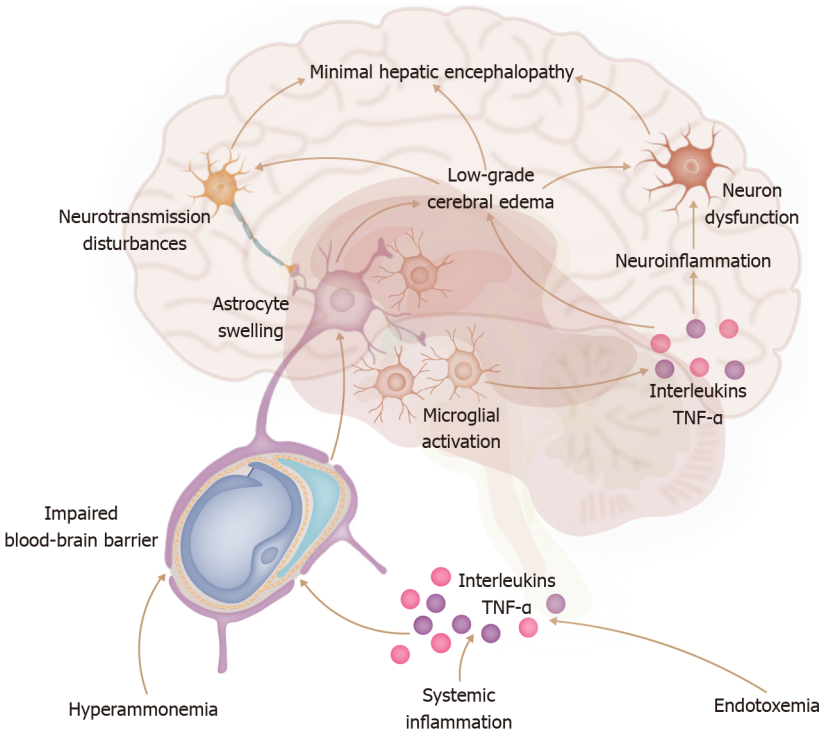
Hyperammonemia

Ammonia, an important causative agent of MHE, is predominantly derived from the degradation of amino acids and urea by the gut bacteria. Urea hydrolysis is catalyzed by urease, an enzyme mainly produced by Gram-negative *Enterobacteriaceae*[49]. In MHE patients, an increased abundance of *Streptococcus salivarius* is associated with hyperammonemia because *Streptococcus salivarius* has a considerable number of urea catabolite genes that activate urease activity, facilitating ammonia production and accumulation, leading to further hyperammonemia[8,29,50]. *Streptococcus salivarius* might be a potential therapeutic target for ammonia-lowering strategies in MHE patients.

Hyperammonemia induces a leaky BBB, promotes glutamine accumulation in astrocytes, and leads to astrocyte swelling and subsequent low-grade cerebral edema that influences neurotransmission (Figure 2)[51,52]. Similar to systemic inflammation, chronic hyperammonemia induces microglial activation with the increased production of TNF-α, IL-1β, and IL-6 and impaired glutamatergic and GABAergic neurotransmission, resulting in cognitive deficits in MHE rats[53,54]. Moreover, treating MHE rats with anti-TNF-α, which does not cross the BBB, attenuated systemic inflammation, alleviated hyperammonemia-induced neuroinflammation, and ameliorated neurotransmission and cognitive function[44]. Experimental animal evidence indicated that hyperammonemia might be exerted in concert with systemic inflammation to drive the development of neuroinflammation.

Endotoxemia

Endotoxins, also known as lipopolysaccharides, are components of the outer membrane of Gram-negative bacteria. In patients with liver cirrhosis, serum endotoxin levels are increased and correlated with MHE severity, and functional modules associated with endotoxin production are abundant in the gut microbiome of MHE patients[11,29]. Several studies reported that increased endotoxin production was related to a higher *Veillonella* abundance in MHE patients[55,56]. Due to the impaired intestinal barrier and portal-systemic shunting, endotoxins enter the systemic circulation and cause endotoxemia with increased production of pro-inflammatory cytokines (Figure 1)[57]. Similar to pro-inflammatory cytokines, endotoxin is also unable to cross the BBB. Nevertheless, endotoxin stimulates microglia to release TNF-α, IL-1β, and reactive oxygen species, which increases the permeability of BBB tight



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Figure 2 Systemic inflammation, hyperammonemia, and endotoxemia influence the permeability of the blood-brain barrier, resulting in neuroinflammation and low-grade cerebral edema, contributing to the pathogenesis of minimal hepatic encephalopathy. TNF-α: Tumor necrosis factor-alpha.

junctions[58]. Peripheral lipopolysaccharide injection induces microglial hyperactivation, increases mRNA expressions of TNF-α, IL-1β, and IL-10 in the cerebral cortex, and impairs glutamate transmission, resulting in memory and learning deficits in mice[59,60]. Based on the synergistic effect of hyperammonemia, peripheral lipopolysaccharide injection induced cytotoxic brain swelling and a subsequent pre-coma status in cirrhotic rats[61]. However, the exact mechanism of the interaction between hyperammonemia and endotoxemia in the pathogenesis of MHE remains unclear and requires further research.

MICROBIOME THERAPEUTICS FOR MHE

The majority of current therapeutic strategies for MHE in clinical practice exert their effects through modulation of gut microbiota dysbiosis. These microbiome therapies, including lactulose, rifaximin, probiotics, synbiotics, and FMT, alter the composition and function of the gut microbiota, inhibit pathogenic bacterial overgrowth, increase the abundance of beneficial bacteria, and reduce the production and absorption of ammonia (Table 2).

Lactulose

Lactulose, the standard therapy for MHE, is considered a prebiotic. A multicenter, randomized, controlled trial in China suggested that lactulose reduces ammonia production and absorption by inhibiting the growth of ammonia-producing bacteria, such as *Streptococcus salivarius*, and facilitates the growth of beneficial saccharolytic bacteria, such as *Bifidobacterium* and *Lactobacillus*[14]. Moreover, several studies have revealed that lactulose reduces the serum concentrations of TNF-α, ILs, and endotoxins by inhibiting SIBO and bacterial translocation, thus improving cognitive dysfunction of MHE patients[11,62,63].

Despite lactulose treatment for MHE patients, there was still an increased gut microbiota dysbiosis with a lower cirrhosis dysbiosis ratio and enriched Gram-negative bacteria such as *Enterobacteriaceae* and *Bacteroidaceae*[64]. Similarly, Sarangi *et al*[65] indicated that lactulose did not significantly influence bacterial diversity, species richness, or taxa abundance in the gut microbiome of cirrhotic patients with MHE. Moreover, lactulose withdrawal only decreased the *Faecalibacterium* abundance and did not remarkably alter the gut microbiota composition[24]. These studies suggest that alterations in the gut microbiota function, rather than changes in the gut microbiota composition, may be associated with the therapeutic effects of lactulose in MHE patients.

Table 2 Clinical Studies of gut microbiota modulation in cirrhotic patients with minimal hepatic encephalopathy

Ref.	Design	Patients	Duration	Sample	Method	Microbiota alteration	Therapeutic effect
Lactulose							
Wang *et al* [14]	Multi-centre, open-label, randomized controlled trial	lactulose (n = 67), control (n = 31)	60 d	Stool	16S rRNA sequencing	Higher abundances of *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Proteobacteria* were in non-responders for lactulose	Significantly ameliorated MHE
Rifaximin							
Bajaj *et al* [56]	Controlled clinical trial	MHE patients before/after rifaximin (n = 20)	8 wk	Stool	Multi-tagged pyrosequencing, GC/LC-MS	Modest decrease in *Veillonellaceae* and increase in *Eubacteriaceae*, with significant changes in metabolite correlations	Significant improvement in endotoxemia and cognition
Probiotics							
Lactobacillus GG							
Bajaj *et al* [74]	Randomized phase I, placebo-controlled trial	Probiotic (n = 14), placebo (n = 16)	8 wk	Stool	Multi-tagged pyrosequencing, GC/LC-MS	Decreased *Enterobacteriaceae* and increased *Lachnospiraceae* and *Clostridiales Incertae Sedis* XIV, with significant alterations in metabolite correlations with amino acid and secondary bile acid metabolism	Attenuated endotoxemia and decreased TNF-α without change in cognition
Probiotics							
Clostridium butyricum combined with *Bifidobacterium infantis*							
Xia *et al* [73]	Randomized controlled trial	Probiotic (n = 30), placebo (n = 37)	3 mo	Stool	16S rRNA sequencing	Increased *Clostridium cluster* I and *Bifidobacterium*, decreased *Enterobacteriaceae* and *Enterococcus*	Reduced ammonia and improved cognition
Escherichia coli Nissle 1917 strain							
Manzhalii *et al*[75]	Single-centre, open-label, randomized trial	Probiotic (n = 15), lactulose (n = 15), rifaximin (n = 15)	1 mo	Stool	16S rRNA sequencing	Normalized *Bifidobacterium* and *Lactobacilli* abundance	Reduced ammonia and pro-inflammatory cytokines and improved cognition
Rifaximin plus probiotic							
Zuo *et al* [84]	Controlled clinical trial	Rifaximin (n = 7), rifaximin plus probiotic (n = 7)	4 wk	Stool	16S rRNA sequencing	Both treatments alone reduced the overall microbiome diversity, with decreased *Streptococcus* and *Faecalibacterium*, *Clostridium* and increased *Lactobacillus*	Rifaximin plus probiotics showed a more apparent effect
Rifaximin plus lactulose							
Schulz *et al*[72]	Randomized controlled trial	Rifaximin (n = 1), rifaximin plus lactulose (n = 4)	3 mo	Stool	16S rRNA sequencing	Rifaximin with or without lactulose did not affect microbiota composition	MHE improvement with rifaximin lasted after the end of treatment
Synbiotics							
Probiotics plus fermentable fiber							
Liu *et al* [76]	Controlled clinical trial	Synbiotic (n = 20), fermentable fiber (n = 20), placebo (n = 15)	30 d	Stool	Stool quantitative bacteriological culture	Significant increase in non-urease-producing *Lactobacillus* species	Reduced ammonia and endotoxemia levels, reversal in 50% of MHE patients

GC/LC-MS: Gas chromatography/liquid chromatography-mass spectrometry; TNF-α: Tumor necrosis factor-alpha; MHE: Minimal hepatic encephalopathy.

Rifaximin

Rifaximin is an oral semisynthetic and nonsystemic antibiotic that inhibits transcription and RNA synthesis by binding to the β-subunit of bacterial RNA polymerase, with lower gastrointestinal absorption and better antimicrobial activity[66]. As an antibiotic, rifaximin also reduced pro-inflammatory cytokines and attenuated systemic and intestinal inflammation in a mouse model of MHE[10]. Similarly, rifaximin-α inhibited serum neutrophil TLR-4 expression, decreased TNF-α and IL levels, and ameliorated MHE in cirrhotic patients[67].

A systematic review and meta-analysis showed that rifaximin is an effective and safe therapy for SIBO with a higher overall eradication rate[68]. In a mouse model of MHE, rifaximin therapy decreased intestinal ammonia production and serum IL-1β and IL-6 Levels by altering the gut microbiota function with increased secondary bile acids and decreased deconjugation without altering the gut microbiota composition[69]. Similarly, several clinical studies revealed that rifaximin attenuated hyperammonemia and endotoxemia in patients with MHE and resulted in significant changes in gut metabolites with modest alterations in gut microbiota composition, such as decreased *Streptococcus* and *Veillonella* abundance[56,70,71]. Furthermore, long-term treatment with rifaximin with or without lactulose did not affect the gut microbiota composition over a period of 3 mo in cirrhotic patients with MHE[72]. The results of these studies further support the theory that rifaximin treats MHE by modulating the metabolic function of the gut microbiota rather than gut microbiota composition, similar to the mechanism of lactulose treatment for MHE.

Probiotics

Probiotics, which are added to yogurt or consumed as food supplements, are live bacteria with various health benefits. Treatment with probiotics containing *B. infantis* and *C. butyricum* increased *Bifidobacterium* and *Clostridium cluster* I abundances and decreased *Enterococcus* and *Enterobacteriaceae* abundances, thereby significantly lowering serum ammonia levels of patients with hepatitis B-associated liver cirrhosis[73]. Additionally, the probiotic *Lactobacillus* GG increased *Clostridiales* XIV and *Lachnospiraceae* abundances, decreased the *Enterobacteriaceae* abundance, and decreased serum endotoxemia and TNF-α levels, resulting in alterations in metabolites associated with amino acid and secondary bile acid metabolism[74]. Moreover, the probiotic *Escherichia coli Nissle* strain reduced the levels of ammonia and pro-inflammatory cytokines, normalized *Lactobacilli* and *Bifidobacterium* abundances, and improved the cognitive function of MHE patients[75]. A systematic review of 19 trials showed that probiotics increased beneficial bacteria such as *Lactobacillus* and *Bifidobacterium*, decreased SIBO and endotoxemia, and reversed MHE without affecting systemic inflammation[15]. Compared with other modalities, including lactulose, rifaximin, and L-ornithine-aspartate, probiotics have similar therapeutic effects on MHE reversal and OHE prevention, and no significant differences were observed in the gut microbiota composition when probiotics and lactulose were compared[15,73]. Probiotics are regarded as alternative therapies for MHE. In contrast to lactulose and rifaximin, probiotics have therapeutic effects on MHE by altering the gut microbiota composition. Because of the complicated interconnections within the gut microbiome, a single change in the gut microbiota composition may have an unexpected effect or no effect at all. The interactions among supplemental probiotics, autochthonous beneficial bacteria, and pathogenic bacteria in the intestinal tract remain to be determined.

Synbiotics

Synbiotics are a combination of probiotics and prebiotics. It is hypothesized that synbiotics improve the effectiveness of probiotics in the human intestine. A synbiotic containing probiotics and fermentable fibers significantly increased the nonurease-producing *Lactobacillus* abundance, decreased serum ammonia levels, and reversed MHE in cirrhotic patients[76]. Moreover, a combination of *Bifidobacterium longum* and fructo-oligosaccharide, which is another symbiotic, decreased serum ammonia levels and improved the cognitive function of MHE patients[77]. A systematic review revealed that synbiotic supplementation decreased SIBO, increased beneficial commensal bacteria such as *Lactobacillus* and *Bifidobacterium*, reduced blood ammonia and endotoxin levels, and decreased the risk of MHE recurrence[15]. Both a synbiotic and a prebiotic alone reduced ammonia and endotoxin levels, decreased the fecal *Escherichia coli* abundance, and reversed MHE; however, the synbiotic did not show better efficacy than the prebiotic alone[76]. Compared with prebiotics and probiotics used alone, the clinical benefits of synbiotics have yet to be demonstrated.

Fecal microbiota transplantation

FMT refers to the process of transferring fecal bacteria from healthy donors to patients with gut microbiota dysbiosis[78]. FMT is an effective therapy for *Clostridioides difficile* infection and inflammatory bowel diseases[78,79]. In germ-free mice colonized with feces from MHE patients, FMT modulated gut microbiota dysbiosis and ameliorated microglial activation and neuroinflammation independent of active liver inflammation[9]. In cirrhotic patients with MHE, oral FMT capsules increased *Ruminococcaceae* and *Bifidobacteriaceae* abundances, decreased *Streptococcaceae* and *Veillonellaceae* abundances, and reduced serum IL-6 and lipopolysaccharide-binding protein[17]. Furthermore, long-term treatment with FMT increased *Burkholderiaceae* abundance and decreased *Acidaminoccocaceae* abundance, which prevented HE recurrence and improved cognitive function during the follow-up[80]. The role of FMT in preventing OHE recurrence by modulating gut microbiota dysbiosis has been demonstrated; however, no clinical trial regarding the FMT for MHE treatment has been reported so far. It could be presumed that FMT is a potential and effective microbiome therapy for MHE; this would require rigorous clinical trials for verification.

Clinical studies of FMT for MHE have used different routes, doses, and dosing times. Owing to these differences, uniform criteria for selecting ideal FMT donors are lacking, and the optimal FMT dosing

regimen remains unclear. Moreover, the identification of pathogens in FMT donors is difficult. FMT is associated with Shigatoxin-producing *Escherichia coli*, extended-spectrum-β-lactamase-producing *Escherichia coli*, and enteropathogenic *Escherichia coli* infections due to the lack of donor screening[81,82]. Patients with liver cirrhosis are vulnerable to infection because of their weakened immune systems; therefore, rigorous screening and selection of FMT donors would improve FMT safety for patients with MHE.

Challenges and controversies

One major challenge of microbiome therapies for MHE is that host factors, dietary habits, and long-term medications may influence the gut microbiome of MHE. *Ruminococcus gnavus* and *Streptococcus salivarius* were the predictors of response to rifaximin treatment, and a higher abundance of *Bacteroidetes*, *Firmicutes*, *Actinobacteria,* and *Proteobacteria* could predict poor response to lactulose treatment[14,83]. Moreover, patients with MHE caused by non-alcoholic cirrhosis responded better to the treatment with rifaximin plus probiotics because they presented a significant decrease in ammonia-producing bacteria genera, such as *Clostridium* and *Streptococcus*[84]. Before the microbiome therapies, the baseline signatures of gut microbiota are identified to match the appropriate microbiome therapies, and gut microbiota biomarkers are explored to predict the therapeutic effects. Based on the different baseline compositions of gut microbiota, targeted and personalized microbiome therapies might be potentially effective strategies for MHE treatment.

Furthermore, cirrhosis is a chronic liver disease, and gut microbiota dysbiosis caused by liver cirrhosis may persist for a long time and require maintenance treatment. However, most microbiome therapeutics are currently single and short-term therapies. After probiotic yogurt supplementation for more than 2 mo, patients with liver cirrhosis had significant MHE reversal rates and excellent compliance; moreover, the potential for long-term compliance existed[85]. Additionally, MHE amelioration with rifaximin treatment for more than 3 mo lasted after the end of treatment, thus indicating a long-term effect on the metabolic function of the gut microbiota[72]. The efficacy and safety of long-term microbiome therapies for MHE require multicenter studies with large populations.

Another related challenge is that rifaximin, a validated antibiotic for MHE, potentially increases antibiotic resistance in liver cirrhosis. A large European cohort study of patients with liver cirrhosis revealed a significant increase in the prevalence of multidrug-resistant bacteria from 29% to 38% during the past decade[86]. Moreover, Chang *et al*[87] reported that rifampin-resistant *staphylococcal* isolates appeared after rifaximin treatment and disappeared during the short term in cirrhotic patients. Prophylactic use of rifaximin did not alter the diversity and composition of gut microbiota or the overall resistance over 12 wk[88]. Rifaximin has not induced significant bacterial resistance and has shown active antimicrobial activity against most bacteria. Multi-drug resistant bacteria should be monitored when using rifaximin in MHE patients, especially in cirrhotic patients previously treated with antibiotics.

CONCLUSION

Gut microbiota dysbiosis initiates the pathophysiological mechanisms of hyperammonemia, systemic inflammation, and endotoxemia, which contribute to neuroinflammation *via* the gut-liver-brain axis in MHE. Currently available strategies for MHE treatment mainly involve the modulation of gut microbiota dysbiosis. In the future, based on the specific microbial signatures identified, personalized and targeted microbiome therapies with optimal regimens and doses may improve the efficacy and safety of MHE treatments.

FOOTNOTES

Author contributions: Luo M and Xin RJ designed the outline, prepared the tables, and drafted the manuscript; Hu FR and Yao L summarized the data and plotted the figure; Hu SJ and Bai FH revised the manuscript. All authors approved the final version of the manuscript.

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Country/Territory of origin: China

ORCID number: Ming Luo 0000-0003-2577-1904; Rui-Juan Xin 0000-0001-6392-9317; Fang-Rui Hu 0000-0002-0222-6279; Li Yao 0000-0002-1120-8435; Sheng-Juan Hu 0000-0002-6210-576X; Fei-Hu Bai 0000-0003-4961-1921.

Corresponding Author's Membership in Professional Societies: Society of Gastroenterology, Chinese Medical Association; Gastroenterologist Branch, Chinese Physicians Association.

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MINIREVIEWS

Endoscopic ultrasound guided radiofrequency ablation for pancreatic tumors: A critical review focusing on safety, efficacy and controversies

Tawfik Khoury, Wisam Sbeit, Bertrand Napoléon

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Tawfik Khoury, Wisam Sbeit, Department of Gastroenterology, Galilee Medical Center, Nahariya 2210001, Israel

Bertrand Napoléon, Department of Endoscopy Unit, Private Hospital Jean Mermoz, Lyon 69008, France

Corresponding author: Tawfik Khoury, MD, Assistant Professor, Doctor, Senior Lecturer, Department of Gastroenterology, Galilee Medical Center, Meona Street, Nahariya 2210001, Israel. tawfikkhoury1@hotmail.com

Abstract

The role of endoscopic ultrasound (EUS) in the last two decades has shifted from a diagnostic tool to an important therapeutic tool treating mainly pancreato-biliary disorders. In recent years, its applications for treating pancreatic diseases have broadened, including the implementation of radiofrequency ablation (RFA), which has been traditionally used for treating solid tumors. In this critical in-depth review, we summarized all the papers throughout the literature regarding EUS-RFA for pancreatic neuroendocrine neoplasms, adenocarcinoma, and pancreatic cystic lesions. Overall, for pancreatic neuroendocrine neoplasms we identified 16 papers that reported 96 patients who underwent EUS-RFA, with acceptable adverse events that were rated mild to moderate and a high complete radiological resolution rate of 90%. For pancreatic adenocarcinoma, we identified 8 papers with 121 patients. Adverse events occurred in 13% of patients, mostly rated mild. However, no clear survival benefit was demonstrated. For pancreatic cystic lesions, we identified 4 papers with 38 patients. The adverse events were mostly mild and occurred in 9.1% of patients, and complete or partial radiological resolution of the cysts was reported in 36.8%. Notably, the procedure was technically feasible for most of the patients. Nevertheless, a long road remains before this technique finds its definite place in guidelines due to several controversies. EUS-RFA for pancreatic tumors seems to be safe and effective, especially for pancreatic neuroendocrine neoplasms, but multicenter prospective trials are needed to consider this treatment as a gold standard.

Key Words: Endoscopic ultrasound; Radiofrequency ablation; Efficacy; Safety; Pancreas; Tumors

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Core Tip: Endoscopic ultrasound guided radiofrequency ablation has been increasingly implemented in the treatment of pancreatic neoplasms. We reviewed the role of endoscopic ultrasound guided radiofrequency ablation in the treatment of pancreatic neuroendocrine tumors, unresectable pancreatic adenocarcinoma, and pancreatic cystic lesions, focusing on efficacy, safety, and controversies. We found that endoscopic ultrasound guided radiofrequency ablation was feasible with an excellent technical success, acceptable adverse events, and a beneficial effect for pancreatic neuroendocrine tumors, mainly on insulinoma. While its effect on pancreatic adenocarcinoma and cystic lesions is promising, more studies are needed to better explore its role.

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INTRODUCTION

In the recent years, endoscopic ultrasound (EUS) transformed from a diagnostic tool to an important therapeutic tool, especially for pancreato-biliary diseases[1]. Among the therapeutic options is radiofrequency ablation (RFA). RFA is a low-risk minimally invasive procedure that delivers heat waves (in the range of 350-500 kHz), with a high temperature ranging between 60 °C-100 °C that subsequently causes burning of the tumorous tissue. The effect is mediated *via* coagulation necrosis, leading to irreversible cellular damage and apoptosis, without significantly affecting the normal surrounding tissue[2]. Safety and effectiveness of EUS-RFA of the pancreatic tissue were evaluated in porcine models, which showed beneficial effects[3-6]. Moreover, RFA should have an anti-cancer effect induced by immunomodulatory activity[7]. RFA was shown previously to be a feasible and safe ablative therapeutic option for liver tumors[8]. With the invention of dedicated needles, RFA has recently been used more under EUS for the treatment of pancreatic neuroendocrine neoplasms (pNENs), pancreatic adenocarcinoma or pancreatic cystic lesions (PCL). However, the data in this field is still emerging. In this current review, we provided a critical in-depth overview of the most updated data of EUS-RFA for pancreatic tumors with a focus on safety, efficacy and controversies.

Literature search

A search for studies published before September 2022 was performed in the PubMed databases with the keywords EUS or endoscopic ultrasound with radiofrequency ablation and any of the following: Pancreatic neuroendocrine tumor or neoplasm, pancreatic functional neuroendocrine tumor or neoplasm, pancreatic non-functional neuroendocrine tumor or neoplasm, insulinoma, carcinoma or adenocarcinoma of pancreas, pancreatic tumor or neoplasm, pancreatic cystic lesions or neoplasms, pancreatic cysts, cysts of the pancreas, mucinous pancreatic cysts, pancreatic serous cystadenoma, intraductal papillary mucinous neoplasm, mucinous cyst, treatment or therapeutic and intervention. The search was restricted to articles in the English language and included prospective, retrospective, randomized controlled studies, case series and case reports. Moreover, the bibliographic section of the selected articles as well as the systematic and narrative articles on the topic were manually searched for further relevant articles. Review articles, presented abstracts and posters, position papers and guidelines were not included. Subsequently, we reviewed and summarized all the data on EUS-guided intervention for solid and cystic pancreatic neoplasms focusing on technical success, safety and efficacy.

Study definitions

Technical success was defined by the successful completion of the procedure (introduction of the RFA probe through EUS channel and induction of thermal current to the pancreatic lesions). Safety was defined by any adverse event (AE) that appeared during the procedure or after and that should be secondary to the EUS-RFA. Efficacy was defined as the complete or partial radiological resolution of the pancreatic tumor. Complete response was defined by total destruction of the lesion, while partial response was defined by 75%-90% destruction of the lesion. The longest follow-up period was used to report efficacy in studies that reported more than one follow-up time point. Pooled data for AE was calculated by the overall number of AE divided by the total number of EUS-RFA sessions. Procedure related AE was defined according to the American Society of Gastrointestinal Endoscopy classification [9] as follow: (1) Mild AE: Post-procedure medical consultation, unplanned hospitalization or hospital

stay prolongation for less than 3 nights; (2) Moderate AE: Unplanned anesthesia, unplanned hospitalization or hospital stay prolongation for 4-10 nights, admission to intensive care unit for 1 night, blood transfusion, interventional radiology or endoscopic treatment for AE secondary to the procedure; and (3) Severe AE: Unplanned admission or hospital stay prolongation for > 10 nights, intensive care stay for > 1 night, surgery needed for an AE related to the procedure, permanent disability and death related to the procedure[9]. The more recent AGREE classification was not used in these trials, and it was not possible retrospectively to find the data that would have been necessary to grade the AE. Pooled radiological response was calculated by the overall number of complete or partial radiological response divided by the total number of patients included. In cases where RFA session numbers were not provided by the original manuscript, we consider it the same as the number of patients included in the study[10-20].

EUS-RFA IN PNENS

To date, most of the studies assessed the role of RFA in the treatment of pNENs, in the form of case reports and case series. The first study was reported by Rossi *et al*[10] on 10 patients with pNENs. RFA was performed by the EUS route in 1 patient with non-functional pNEN. Lakhtakia *et al*[12] reported the first case series of 3 patients with functional pNENs (insulinoma), with rapid hypoglycemia relief in the same day. Similarly, Waung *et al*[21] and Bas-Cutrina *et al*[22] reported 2 successful cases of EUS-RFA for insulinoma. Pai *et al*[23] reported a study including 8 patients with complete resolution at 3-6 mo post-treatment and no procedure-related AEs.

Barthet *et al*[13] reported the first multicenter prospective study including 12 patients with 14 pNENs who underwent RFA. Two patients developed complications (16.7%), including acute pancreatitis with early infected necrosis and main pancreatic duct stenosis. Notably, the patient who developed infected necrosis had a cystic pNEN, and the cystic component was not aspirated before performing the RFA. Therefore, this AE was presumed to be secondary to the lack of cystic component suction. After this AE, the independent safety committee decided to administer antibioprophylaxis (2 g of intravenous amoxicillin and clavulanic acid intravenously) and to aspirate the main part of the fluid content prior to RFA in cystic pNEN in order to avoid excessive application of radiofrequency current into the liquid component[13]. The long-term follow-up data of the study by Barthet *et al*[24] was published recently; among the 12 patients with the 14 pNENs lesions, there was a complete disappearance in 12 pNENs lesions (85.7%) and 2 failures (14.3%) at a mean follow-up of 45.6 mo. The two failures were pNEN recurrence after disappearance at 1 year and a metastatic evolution at 3 years follow-up in a patient that had a persistence of the initial pancreatic tumor after two RFA sessions.

Another study by Choi *et al*[25] reported 8 patients with pNENs. Notably, the proliferative index Ki67% was reported in only 2 patients (1 patient with G1 and one patient with G2). Similarly, a prospective study by de Nucci *et al*[26] reported a complete radiological resolution rate at 6 and 12 mo following treatment among 10 patients with 11 pNEN lesions of G1 grading (< 5%), with only 2 mild AEs. Oleinikov *et al*[14] reported a retrospective study that included 18 patients with pNENs. Two patients with non-functional pNEN and one patient with insulinoma had multiple endocrine neoplasia syndrome type 1. Most of the lesions were G1 grading. Complete relief of hypoglycemia-related symptoms was achieved in the 7 patients with insulinoma within 1 h following the EUS-RFA[14].

Furthermore, recent case reports and prospective case series were published in patients with insulinoma who underwent EUS-RFA, with a complete clinical resolution of the hypoglycemic symptoms up to 1 d after the EUS-RFA and complete radiological resolution[27-29], with 1 case of acute necrotizing pancreatitis[28] and 2 cases of mild pancreatitis occurring 1 d after the procedure and 3 mo after[29]. Rossi *et al*[30] treated 3 elderly patients with insulinomas with one intraprocedural bleeding treated endoscopically.

Additionally, Marx *et al*[31] reported two recent trials. The first study included 7 patients with insulinoma, with a complete resolution rate reported in 6 patients (85.7%). Clinical success in terms of symptom relief was achieved immediately in all patients (100%). Notably, this study was associated with a safety signal, as 3 patients had mild to moderate AEs and 1 patient (aged 97 years) had a severe AE with retrogastric collection. He refused drainage, was symptomatically treated and died 2 wk later [15]. The second study retrospectively reported 27 patients with G1 non-functional pNENs. Nine out of the 27 lesions (33.3%) were cystic. Twenty-five patients (92.6%) had a complete radiological resolution at a mean follow-up time of 15.7 mo (range 2-41). Notably, procedure-related AEs occurred in 9 patients [31] (Table 1).

Pooling the available data, EUS-RFA was performed on 100 patients with 112 pNEN lesions that underwent 114 EUS-RFA sessions. Most of the data were published as case reports and small case series. The mean lesion size was 14.8 mm, ranging mostly from 10-20 mm. The procedure was technically feasible in all patients, and the AE rate was almost 21.9%, occurring in 25 of the 114 EUS-RFA sessions. Notably, most of the AE were mild and moderate according to the American Society of Gastrointestinal Endoscopy guideline[9] except for one fatal AE in a recent paper published by Marx *et al*[15]. Interestingly, the complete radiological resolution rate was approximately 90% during a follow-up

Table 1 Studies reporting endoscopic ultrasound-radiofrequency ablation for pancreatic neuroendocrine neoplasms

Ref.	Type of study (pNEN type)	Patients/lesions/RFA sessions, *n*	Location, *n*	Mean size (range) in mm	Histological grade (KI67%)	Technical success, *n* (%)	Adverse events, *n* (%)	Complete radiological/ clinical[1] resolution, *n* (%)	Mean follow-up in mo
Rossi *et al* [10]	Case report (nonfunctional)	1/1/1	Head (1)	10	NR	1 (100)	0	1 (100)	34
Armellini *et al*[11]	Case report (nonfunctional)	1/1/1	Tail (1)	20	G2 (> 5)	1 (100)	0	1 (100)	1
Lakhtakia *et al*[12]	Case series (insulinoma)	3/3/3	Body (2), diffuse (1)	17.7 (14-22)	NR	3 (100)	0	3 (100)/3 (100)	4.2
Waung *et al*[21]	Case report (insulinoma)	1/1/3	Uncinate (1)	18	NR	1 (100)	0	1 (100)/1 (100)	10
Bas-Cutrina *et al*[22]	Case report (insulinoma)	1/1/1	Body (1)	10	NR	1 (100)	0	1 (100)/1 (100)	10
Pai *et al* [23]	Prospective (nonfunctional)	2/2/3	Head (1)	27.5 (15-40)	NR	2 (100)	0	2 (100)	6
Barthet *et al*[13]	Prospective (nonfunctional)	12/14/12	Head (3), body (6), tail (5)	13.1 (10-20)	G1	12 (100)	2 (16.7)[2]	9 (75)	12
Choi *et al* [25]	Prospective (nonfunctional-7), (insulinoma-1)	8/8/14	Head (3), body (5)	19.25 (8-28)	Reported in 2 patients (G1 and G2)	8 (100)	2 (14.3)[3]	6 (75)/1 (100)	13
de Nucci *et al*[26]	Prospective (nonfunctional)	10/11/10	Head (3), body (8), tail (2)	14.5 (9-20)	G1 (< 4)	10 (100)	2 (20)[4]	10 (100)	12
Oleinikov *et al*[14]	Retrospective (nonfunctional-11), (insulinoma-7)	18/27/18	Head (10), body (8), tail (2), uncinate (5), metastasis (2)	14.8 (12-19)	G1 (< 5) in 15 patents, G3 (34-40) in 2 patients	18 (100)	2 (11.1)[5]	17 (94.4)/7 (100)	8.7
Rossi *et al* [30]	Case series (insulinoma)	3/3/4	NR	11.5 (9-14)	NR	3 (100)	1 (25)[6]	3 (100)/3 (100)	8.5
Chang *et al*[27]	Case report (insulinoma)	1/1/1	Head (1)	12	NR	1 (100)	0	1 (100)/1 (100)	18
Kluz *et al* [28]	Case report (insulinoma)	1/1/1	Head (1)	9	NR	1 (100)	1 (100)[7]	NR/1 (100)	NR
Furnica *et al*[29]	Case series (insulinoma)	4/4/4	Head (2), neck (1), tail (1)	12.9 (6.5-22.0)	G1 in 3 patients and G2 in 1 patient	4 (100)	2 (50)[8]	4 (100)/4 (100)	22
Marx *et al* [15]	Retrospective (insulinoma)	7/7/7	Head (1), body (1), neck (3), body-tail junction (2)	13.3 (8-20)	G1 (< 3) in 4 patient, G2 (4) in 1 patient	7 (100)	4 (57.1)[9]	6 (85.7)/7 (100)	20.3
Marx *et al* [31]	Retrospective (non-functional)	27/27/31	Head (6), body (3), tail (11), uncinate (2), body-tail junction (5)	14 (7-25)	G1 (< 3) in 25 patients, NR in 2 patients	27 (100)	9 (29)[10]	25 (92.6)	15.7
Pooled data	Case reports: 9. Prospective: 4. Retrospective: 3	100/112/114	Head and neck (33), body (34), tail (22), uncinate (8), metastasis and diffuse (3), junction (7)	14.8	Unable to pool due to data lacking	96 (100)	25 (21.9)	90 (90)/21 (100)	13

[1]Insulinoma.

[2]Infected pancreatic necrosis (1 patient, moderate), pancreatic duct stenosis (1 patient, moderate).
[3]Pancreatitis (1 patient, moderate), transient abdominal pain (1 patient, mild).
[4]Transient abdominal pain (2 patients, mild).
[5]Pancreatitis (2 patients, mild).
[6]Intraprocedural bleeding treated endoscopically (1 patient, moderate).
[7]Acute necrotizing pancreatitis (1 patient, moderate).
[8]Pancreatitis (2 patients, mild).
[9]Transient abdominal pain (1 patient, mild), pancreatitis (1 patient, moderate), coagulation necrosis (1 patient, moderate), retrogastric collection (1 patient, refused drainage, died 2 wk later, severe).
[10]Transient abdominal pain (3 patients, mild), pancreatitis (1 patient, moderate), periprocedural bleeding (2 patients, moderate), pancreatitis complicated by retrogastric or perisplenic collection managed by endoscopic drainage and antibiotic treatment (2 patients, moderate), pancreatitis with pancreatic fistula and paragastric collection drained by endoscopic cystgastrostomy (1 patient, moderate).
pNEN: Pancreatic neuroendocrine neoplasms; NR: Not reported; RFA: Radiofrequency ablation.

period of 13 mo (Table 1).

EUS-RFA IN PANCREATIC ADENOCARCINOMA

Recently, EUS-RFA was increasingly implemented in the treatment of pancreatic adenocarcinoma among patients who were not candidates for surgical resection. The first study was a feasibility study conducted by Arcidiacono *et al*[16] who reported 22 patients with locally advanced pancreatic adenocarcinoma who underwent EUS-RFA. Before the EUS-RFA treatment, all patients had received gemcitabine-based chemotherapy, and 6 patients had chemoradiation. Data regarding chemotherapeutic and radiation-induced response were available in 16 patients (3 patients had a partial response, whereas 13 had stable disease). The procedure was technically successfully completed in 16 patients (72.7%). For 6 patients, there was a failure to penetrate the gastric wall and the tumor. The number of procedure-related AEs was relatively high and noted in 8 patients (36.4%). However, most of them were mild. Neither clear survival benefit nor significant effect on tumor size was evidenced[16].

Later, Song *et al*[32] reported the safety among 6 patients with pancreatic adenocarcinoma (4 patients with locally advanced disease and 2 patients with metastatic disease). Three patients were on adjuvant chemotherapy with gemcitabine, whereas the other 3 patients did not receive concomitant chemotherapy. The procedure was successfully completed in all patients with only 2 mild procedure-related AEs (mild abdominal pain)[32]. Scopelliti *et al*[17] reported 10 patients with locally advanced pancreatic adenocarcinoma. All patients underwent systemic chemotherapy (4 patient received FOLFIRINOX, 2 patients received gemcitabine, 2 patients received GemOx and 2 patients received combined gemcitabine/nab-paclitaxel), and 5 patients underwent additional external radiation therapy. All patients had complete technical success, and mild pancreatitis occurred in 4 patients, with no major AEs[17].

Similarly, Crinò *et al*[18] reported 7 patients with locally advanced pancreatic adenocarcinoma that were previously treated with FOLFIRINOX + radiotherapy (3 patients), gemcitabine (2 patients), FOLFIRINOX (1 patient) and radiotherapy (1 patient) who underwent EUS-RFA with an excellent technical success rate and minor AE of mild abdominal pain in 3 patients. Mean tumor ablation was approximately 30% (5.8%-73.5%) at 30 d following the procedure. However, data regarding survival benefit were not reported[18]. Paiella *et al*[19] reported a genetic study of 30 patients with locally advanced adenocarcinoma. Thirteen patients received EUS-RFA before the chemotherapy, while 17 patients had EUS-RFA after treatment (FOLFIRINOX in 6 patients, gemcitabine/oxaliplatinum in 4 patients, nab paclitaxel/gemcitabine in 2 patients and data not available in 5 patients, with additional radiotherapy in 4 patients). The overall median disease specific survival for all patients was 15 mo. *SMAD4* mutation was diagnosed in 18 patients (60%). The estimated post-RFA disease specific survival of patients without and with *SMAD4* mutation was 22 mo and 12 mo, respectively, with complete technical success of EUS-RFA and only 1 AE of bleeding from a duodenal ulcer[19].

Moreover, a recent prospective randomized study by Bang *et al*[20] reported the yield of EUS-guided RFA (12 patients) *vs* celiac plexus neurolysis (14 patients) for palliation of pain in pancreatic adenocarcinoma. EUS-RFA guided treatment was associated with a significant improvement in pain associated with pancreatic cancer ($P < 0.05$). Procedure-related AE occurred in 10 out of 12 included patients (83.3%) but were always mild[20]. Another recent study by Wang *et al*[33] reported 11 patients with pancreatic adenocarcinoma (only 1 patient was on chemotherapy), with complete technical success and only 2 patients with minor AEs of abdominal pain. A decrease in tumor size was only notable in 2 patients (18.2%), without a significant benefit on survival[33]. A recent study by Oh *et al*[34] reported 22 patients with pancreatic adenocarcinoma (19 patients received systemic gemcitabine-based chemotherapy before, and 3 patients received chemotherapy) who underwent 107 EUS-RFA sessions. The overall survival rate was 24 mo, with 4 procedure-related AEs (3 patients had transient abdominal pain, and 1 had peritonitis)[34].

Overall, the pooled analysis showed that EUS-RFA was applied to date in 120 patients with pancreatic adenocarcinoma who underwent 222 EUS fine needle aspiration sessions, most of them with locally advanced disease. The mean lesion size was 37.4 mm. The procedure was successfully completed in 95% of the patients, and AE occurred in 29 EUS-RFA sessions (13%), most of them were mild in severity, including transient abdominal pain and gastrointestinal symptoms. Notably, any decrease in tumor size was reported in 4 studies, as it was recorded in 25 among 50 patients (50%). However, only 4 studies provided data regarding the post EUS-RFA survival. Two studies did not show a clear survival benefit[16,33], and the other two studies showed a potential survival benefit[19,34] (Table 2).

RFA IN PCL

In the last few years, EUS fine needle aspiration was also implemented in the treatment of PCL in a few human case series. The first case was reported by Wiersema *et al*[35] in a patient with bleeding remnant intraductal papillary mucinous neoplasm that was successfully treated with endoscopic intraductal RFA. Pai *et al*[23] prospectively reported 6 patients with PCL [4 mucinous cystic neoplasm, 1 intraductal papillary mucinous neoplasm and 1 serous cystadenoma (SCA)]. Two (33.3%) and four (66.7%) patients had complete and partial cyst resolution at 3-6 mo follow-up, respectively. Among the 4 patients with partial resolution, 2 patients (50%) had > 50% ablation of the cyst size. Only 2 patients (33.3%) had mild transient abdominal pain. Notably, no long follow-up data were provided to assess recurrence. Furthermore, Choi *et al*[25] reported 2 patients with solid pseudopapillary tumors who underwent EUS-RFA because they refused surgery. The procedure was successfully completed in both patients, without procedure-related AEs. At a median follow-up of 13 mo, 1 patient (50%) had complete radiological response, while the other patient had no response with a decrease of approximately 20% from its pre-ablation size[25].

Additionally, Barthet *et al*[13] reported the yield of EUS-RFA among 17 patients with PCL (16 patients with intraductal papillary mucinous neoplasm and 1 patient with mucinous cystic neoplasm), notably 12 patients (70.6%) and 4 patients (23.5%) had mural nodules and thick cystic walls, respectively. The follow-up was assessed at two time-points. At 6-mo, 8 patients (47.1%) had a complete disappearance and necrosis of the cysts, and 3 patients (17.6%) had > 50% decrease in cyst diameter. However, there were 6 patients (35.3%) with failure of the procedure. At 12-mo follow-up, 11 patients (64.7%) had a complete disappearance and necrosis of the cysts, and 1 patient (5.9%) had > 50% decrease in cyst diameter. However, there were 5 patients (29.4%) with procedure failure. Only 1 procedure-related AE was noted with fever and pneumoperitoneum due to a perforation of the jejunal loop surgically corrected[13]. The long-term follow-up in 15 patients was recently reported. At 42.6-mo follow-up, complete cyst disappearance was noticed in 6 patients (40%). Four patients (26.6%) had a partial radiological response (decrease > 50% of the initial cyst diameter). Failure was seen in 5 patients, as the cyst lesion decreased < 50%[24].

A recent study by Oh *et al*[36] reported 13 patients with SCA who underwent 19 EUS-RFA sessions. One patient (5.3%) had peri-procedural transient mild abdominal pain. Notably, none of the patients had complete radiological response at 9.2 mo of follow-up, while 8 patients (61.5%) had partial radiological response (more than 30% in the longest diameter with an estimated volume reduction more than 66%)[36].

Pooling the data, 4 studies assessed EUS-RFA for PCL, with 38 patients included who underwent 44 EUS-RFA sessions. The mean cyst size was 32.1 mm, and worrisome features were only reported in one study. The procedure was feasible in all patients, with mild AEs of transient abdominal pain in most studies. Notably, complete radiological cyst resolution was achieved in 14 patients (36.8%) at a follow-up of 10.2 mo (Table 3).

SAFETY AND EFFICACY OF EUS-RFA IN PANCREATIC TUMORS

Overall, 377 EUS-RFA sessions were performed in 255 patients. The rate of mild, moderate and severe AEs according to American Society of Gastrointestinal Endoscopy guidelines[9] were 10.1%, 4.2% and 0.5%, respectively. For pNENs, the rate of mild and moderate AEs was 8.2% and 11.8%, respectively. For pancreatic adenocarcinoma and pancreatic cystic tumors, most of the AE were mild in severity. Notably, the rate of severe AEs and mortality were extremely low in all pancreatic tumor categories (Table 4). Finally, the EUS-RFA treatment is technically feasible, with high clinical and radiological success rates for pNENs and PCL and an acceptable AE rate (Table 5). Nevertheless, some limitations and controversies must be underlined as those limitations might impact the interpretation of the published literature and should be considered when planning future studies.

Technical considerations

The studies reported different power setting and application number used (Table 6). Moreover, in

Table 2 Studies reporting endoscopic ultrasound-radiofrequency ablation for pancreatic adenocarcinoma

Ref.	Study type	Patients/RFA session, *n*	Tumor location (*n*)	Cancer stage (*n*)	Mean size (range) in mm	Any decrease in tumor size, *n* (%)	Technical success, *n* (%)	Adverse events, *n* (%)	Mean follow-up in mo	Survival after RFA in mo
Arcidiacono *et al*[16]	Prospective	22/22	Head (16), uncinate (2), body (4)	Locally advanced (22)	35.7 (23-54)	6 (37.5)	16 (72.7)	8 (36.4)[1]	3	5.6 (1-12)
Song *et al* [32]	Prospective	6/8	Head (4), body (2)	Locally advanced (4), metastasis (2)	48 (30-90)	NR	6 (100)	2 (25)[2]	4.2	NR
Scopelliti *et al*[17]	Prospective	10/10	Head (4), body (6)	Locally advanced (10)	49.2 (25-75)	10 (100)	10 (100)	4 (40)[3]	1	NR
Crinò *et al* [18]	Retrospective	7/7	Head (2), body (3), uncinate (2)	Locally advanced (7)	36 (22-67)	7 (100)	7 (100)	3 (42.8)[4]	6.1	NR
Paiella *et al* [19]	Retrospective	30/30	Head (23), body and tail (7)	Locally advanced (30)	35 (20-60)	NR	30 (100)	1 (3.3)[5]	15	15
Bang *et al* [20]	Prospective	12/12	Head and uncinate (8), body and tail (4)	Locally advanced (5), metastasis (7)	29.6 (22.5-35.0)	NR	12 (100)	5 (41.6)[6]	1	NR
Wang *et al* [33]	Retrospective	11/26	Head (4), neck (3), body (3), tail (1)	Locally advanced (7), metastasis (4)	28 (17.2-38)	2 (18.2)	11 (100)	2 (7.7)[7]	5.2	5.2
Oh *et al*[34]	Prospective	22/107	Head (14), body (4), tail (3), metastasis (1)	Locally advanced (14), metastatic (8)	38 (32.8-45.0)	NR	22 (100)	4 (3.7)[8]	21.2	24
Pooled data	Prospective: 5. Retrospective: 3	120/222	Head and uncinate (79). Body and tail (37), neck (3)	Locally advanced (100), metastasis (21)	37.4	Unable to pool due to data lacking	114 (95)	29 (13)	7.1	Unable to pool due to data lacking

[1]Transient abdominal pain (3 patients, mild), minor duodenal bleeding endoscopically treated (1 patient, moderate), transient amylase elevation (3 patients, mild), transient cystic fluid collection between pancreas and left hepatic lobe (1 patient, mild).
[2]Transient abdominal pain (2 patients, mild).
[3]Mild pancreatitis (4 patients, mild).
[4]Transient abdominal pain (3 patients, mild).
[5]Duodenal bleeding (1 patient, moderate).
[6]Nausea and vomiting (4 patients, mild), transient abdominal pain (1 patient, mild).
[7]Transient abdominal pain (2 patients, mild).
[8]Transient abdominal pain (3 patients, mild), peritonitis (1 patient, moderate).
NR: Not reported; RFA: Radiofrequency ablation.

several studies, the size of the tip of the needle was not considered or not detailed. Power setting, size of the active type, duration of the irradiation, size of the needle (18 G *vs* 19 G) can interfere in the final destruction. Therefore, uniform studies with similar technical aspects should be performed to better assess the treatment efficacy and safety.

Optimal size of the pNENs and PCL

To date, no data are available regarding the optimal size of the pNENs and cystic lesions that are amenable to EUS-RFA. Predictably, the RFA probe can induce a 3 cm ablation area with a single deployment, thus it is postulated that lesions up to 3 cm will achieve the best ablative results with a single application, and larger lesions may need more needle applications during the same session[6]. In fact, a lot of lesions had more than one needle application during the same session even in lesions < 2

Table 3 Studies reporting endoscopic ultrasound-radiofrequency ablation for pancreatic cystic tumors

Ref.	Type of study	Patients/RFA sessions, *n*	Type of cyst	Mean size (range) in mm	Worrisome features	Technical success, *n* (%)	Adverse events, *n* (%)	Complete/partial resolution, *n* (%)	Mean follow-up in mo
Pai *et al* [23]	Prospective	6/6	MCN (4), IPMN (1), MCA (1)	41 (24-70), 35, 20	NR	6 (100)	2 (33.3)[1]	2 (33.3)/4 (66.7)	6
Choi *et al*[25]	Prospective	2/2	SPT (2)	21.5 (20-23)	NR	2 (100)	0	1 (50)/1 (50)	13
Barthet *et al*[13]	Prospective	17/17	MCN (1), IPMN (16)	28 (9-60)	16 (94.1)	17 (100)	1 (5.9)[2]	11 (64.7)/1 (5.9)	12
Oh *et al* [36]	Retrospective	13/19	SCN (13)	50 (34-52.5)	NR	13 (100)	1 (5.3)[3]	0/8 (61.5)	9.2
Pooled data	Prospective: 3. Retrospective: 1	38/44	MCN (5), IPMN (17), MCA (1), SPT (2), SCN (13)	32.1	Unable to pool due to data lacking	38 (100)	4 (9.1)	14 (36.8)/14 (36.8)	10.2

[1]Transient abdominal pain (2 patients, mild).
[2]Fever and pneumoperitoneum with fluid collection and jejunal loop perforation needed surgery (1 patient, severe).
[3]Transient abdominal pain (1 patient, mild).
IPMN: Intraductal papillary mucinous neoplasm; MCA: Microcystic adenoma; MCN: Mucinous cystic neoplasm; NR: Not reported; RFA: Radiofrequency ablation; SPT: Solid pseudopapillary tumor; SCN: Serous cystic neoplasm.

Table 4 Pooled analysis of the adverse events

EUS-guided RFA for	Procedure-related adverse events according to ASGE[9][1]			
	Mild, *n* (%)	Moderate, *n* (%)	Severe, *n* (%)	Mortality, *n* (%)
Neuroendocrine neoplasms EUS-RFA sessions = 114	11 (9.6)	13 (11.4)	1 (0.9)	1 (0.9)
Adenocarcinoma EUS-RFA sessions = 223	26 (11.6)	3 (1.3)	0	0
Cystic tumors EUS-RFA sessions = 44	3 (6.8)	0	1 (2.3)	0
Pooled data	40 (10.5)	16 (4.2)	2 (0.5)	1 (0.26)

[1]Overall, 380 EUS-RFA sessions performed for all pancreatic tumors.
ASGE: American Society of Gastrointestinal Endoscopy; EUS-RFA: Endoscopic ultrasound-radiofrequency ablation.

cm.

Heterogenicity of reporting the histological grading and mitotic activity for the pNENs

EUS-RFA for pNENs should be reserved for patients with G1 (Ki67 < 3%) or low G2 (Ki67 < 5%). However, most of the reported studies did not address the histological and mitotic activity of the pNENs, and in one study by Oleinikov *et al*[14], 2 patients with G3 (Ki67% of 34%-40%) were included in their series. Therefore, identification of the optimal histological grading that will most benefit from EUS-RFA is needed.

Technical success

In the published papers, the technical success was almost complete. However, the data did not state how many patients failed to undergo the procedure due to technical difficulties. Thus, the pooled technical success rate should be carefully interpreted. Further prospective studies are warranted with inclusion of all patients referred for an EUS-RFA procedure in intention-to-treat.

AE rate

Most of the AEs that were reported in the literature were intra- and periprocedural AEs, mainly reported from retrospective and small series with scarce data on long-term AEs (follow-up of only 1 mo for some trials). Moreover, there was one death in an elderly patient who refused endoscopic intervention, which might bias the severity of AEs as well. Therefore, larger studies are needed with

Table 5 Summary of efficacy and safety of endoscopic ultrasound-guided radiofrequency ablation for pancreatic tumors

Procedure	EUS-guided RFA for pancreatic		
	Neuroendocrine tumors	Adenocarcinoma	Cystic tumors
Technical success	High	High	High
Safety, complications	Mild-moderate[1]	Mild	Mild
Efficacy			
Clinical improvement	Significant for insulinomas	None	-
Radiological partial/complete resolution	High	Modest	Encouraging
Palliation	-	Encouraging	-
Mortality	None	None	None

[1]When taking prophylactic measures (antibioprophylaxis, fluid component suction before radiofrequency ablation).
EUS-RFA: Endoscopic ultrasound-radiofrequency ablation.

Table 6 Technical considerations and imaging studies used in follow-up among patients with pancreatic neuroendocrine neoplasms

Ref.	Number of patients/sessions	Power setting in Watts	RFA application number in all sessions	Imaging study used in radiological follow-up
Rossi *et al*[10]	1/1	10-15	1	CECT or MRI
Armellini *et al*[11]	1/1	NR	2	CT and CE-EUS
Lakhtakia *et al*[12]	3/3	50	9	CECT (1 patient), CECT and EUS (2 patients)
Waung *et al*[21]	1/3	10	25	CT and gallium dotatate positron emission tomography
Bas-Cutrina *et al*[22]	1/1	10	3	NR
Pai *et al*[23]	2/3	20	10	Cross sectional imaging not stated which
Barthet *et al*[13]	12/12	50	NR	CT and EUS
Choi *et al*[25]	8/14	50	65	CECT and CE-EUS
de Nucci *et al*[26]	10/10	20	23	CT
Oleinikov *et al*[14]	18/18	50	3-10 in each EUS-RFA session	CECT (9 patients), NA (5 patients), CECT and somatostatin receptor imaging (3 patients)
Rossi *et al*[30]	3/4	30	14	EUS (1 patient), MRI (1 patient), refused follow-up (1 patient)
Chang *et al*[27]	1/1	50	2	CECT
Kluz *et al*[28]	1/1	50	3	NR
Furnica *et al*[29]	4/4	50	1-3 per each EUS-RFA	CT
Marx *et al*[15]	7/7	50	1-5 for each EUS-RFA session	CE-EUS or MRI
Marx *et al*[31]	27/31	50	1-5 for each EUS-RFA session	CT or MRI

CE-EUS: Contrast enhanced endoscopic ultrasound; CECT: Contrast enhanced computed tomography; CT: Computed tomography; EUS-RFA: Endoscopic ultrasound-radiofrequency ablation; MRI: Magnetic resonance imaging; NA: Not available; NR: Nor reported.

longer follow-up to better define the AEs in these procedures.

Antibioprophylaxis in cystic lesions

Antibioprophylaxis and liquid component suction of all the fluid composition of the lesions before performing the RFA procedure is a controversy that should be addressed for cystic pNENs and for PCL. In their study, Barthet *et al*[13] revised their prophylaxis protocol after an AE of infection, so they administered antibioprophylaxix and aspirated the major cystic liquid component in their subsequent patients. Antibioprophylaxis in a PCL patient who underwent EUS-guided fine needle aspiration has

been a long debated clinical indication, as there were conflicting results regarding this condition[35,37-39]. A recent meta-analysis showed no significant difference in the rate of pancreatic cyst infection rate after puncture irrespective of the administration of antibioprophylaxis[40]. Moreover, the advantage of emptying the cyst might be a double pitfall. It will be less evident to see the thickening or the mural nodule within the PCL undergoing EUS-RFA, and it will need two punctures (one for emptying the fluid, and one for the EUS-RFA procedure), which might increase the procedure-related AE.

Association between complete clinical and radiological resolution in insulinomas

The complete disappearance of the clinical symptoms of insulinoma occurred in all patients (100%) throughout the reported studies. However, it does not mean that the tumor was totally destroyed, as some patients with insulinoma will have normal insulin levels[41,42]. Among the nine studies that included patients with insulinoma, only three studies had almost similar clinical and radiological follow-up periods after EUS-RFA, while the other studies had a longer clinical than radiological follow-up (Table 7). Further prospective studies are needed with uniform clinical, biochemical and radiological long-term follow-up periods.

Radiological efficacy

According to the literature, a high complete radiological resolution rate was demonstrated after EUS-RFA. However, the studies reported different imaging modalities or combined imaging tools. Moreover, some studies did not specify which imaging tool was used. Notably, only three studies used a combination of contrast enhanced computed tomography (CT) and contrast enhanced EUS, while most of the other studies used only single imaging modality. Furthermore, in some studies, CT and EUS were used for follow-up. However; it was not stated whether contrast enhancement was implemented (Table 6).

Previous studies have shown that contrast enhanced magnetic resonance imaging including diffusion-weighted imaging is preferred over contrast enhanced CT for examination of the pancreas and the liver[43,44]. On the other hand, EUS has an important role in the diagnosis of small pNENs of < 2 cm and is now considered as the imaging study of choice to be performed where other non-invasive studies failed to diagnose the pNENs[45,46]. Previous systematic review and meta-analysis showed that EUS consistently increased the detection of pNENs by over 25% after performing CT scan[47]. PET-Dotatoc should also be proposed for the follow-up of non-functional pNENs. Therefore, a prospective study with uniform imaging study to be used at follow-up is mandatory to precisely assess the efficacy of EUS-RFA in pNENs.

Patient number and study designs

The small number of patients reported and the study designs, which were primarily case reports and small case series, with the lack of uniform and long-term follow-up should urge careful interpretation of the current literature. The follow-up is too short (only one trial has a follow-up longer than 3 years) to know the long-term result on the tumor and on the possible metastatic evolution.

RFA in PCL

The indication of RFA in cystic lesions remains debated. Oh *et al*[36] reported a study on 13 patients with SCA. However, the interest in this indication is debatable due to its very rare malignant potential [48]. Excluding SCA, only 25 patients with PCL were treated by EUS-RFA, which is too small of a sample size to enable good and precise data interpretation. Therefore, more studies are needed in patients with high-risk PCL.

RFA in pancreatic adenocarcinoma

Most of the studies did not report the additional survival benefit of EUS-RFA when added to standard chemotherapeutic regimens. Moreover, some studies included patients with metastatic disease. It is difficult to justify this treatment for metastatic disease. Prospective randomized trials with uniform disease stage and standard chemotherapeutic regimens are necessary to draw conclusions of the efficacy.

CONCLUSION

High and promising expectations are held for EUS-RFA. Taking advantage of the EUS transducer proximity to the pancreatic parenchyma, coupled with its excellent imaging resolution and the capability of avoiding major internal organs and vascular structures, makes this procedure safe. The current evidence of efficacy is weak, as most studies were case reports and series that included a small number and heterogenous groups of patients. Prospective and randomized studies are needed to establish the potential therapeutic role of EUS-RFA in pancreatic tumors. The available literature suggests a beneficial impact mainly on functional pNENs where RFA should replace surgery. In nonfunctional pNENs the data are encouraging. Its role for PCL treatment is still to be elucidated. For

Table 7 Clinical and radiological follow-up in pancreatic insulinomas studies

Ref.	Patients with insulinoma, *n*	Mean time (range) of clinical follow-up in mo	Mean time (range) of radiological follow-up in mo
Lakhtakia *et al*[12]	3	11.7 (11-12)	4.2 (1.5-8)
Waung *et al*[21]	1	10	3 d
Bas-Cutrina *et al*[22]	1	10	10
Choi *et al*[25]	1	NR	NR
Oleinikov *et al*[14]	7	9.7 (3-21)	8.7 (2-21)
Rossi *et al*[30]	3	22 (14-27)	5.7 (3-14)
Chang *et al*[27]	1	18	18
Kluz *et al*[28]	1	NR	NR
Furnica *et al*[29]	4	22 (13-28)	8 (3-14)
Marx *et al*[15]	7	21 (3-38)	20.3 (3-38)

NR: Not reported.

pancreatic adenocarcinoma, the data are lacking especially on the survival rate. Finally, EUS-RFA for pancreatic tumors is far from being adopted as a first-line treatment except for insulinomas. For grade 1 nonfunctional pNENs < 2 cm, EUS-RFA should be discussed as an alternative to surgery or follow-up. For PCL with worrisome features, EUS-RFA could be considered among patients who are not candidates or refuse surgical intervention. For pancreatic adenocarcinoma, randomized controlled trials are required to determine if EUS-RFA adds a survival benefit to chemotherapy in locally advanced pancreatic adenocarcinoma.

FOOTNOTES

Author contributions: Napoléon B and Khoury T contributed to study design; all authors contributed to the data collection; Napoléon B and Khoury T contributed to the critical revision of the manuscript; and all authors approved the final version to be published.

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Country/Territory of origin: Israel

ORCID number: Tawfik Khoury 0000-0001-6699-8625; Wisam Sbeit 0000-0002-0921-4676; Bertrand Napoléon 0000-0001-5866-8132.

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ORIGINAL ARTICLE

Basic Study

In vivo recognition of bioactive substances of *Polygonum multiflorum* for protecting mitochondria against metabolic dysfunction-associated fatty liver disease

Li-Ping Yu, Yan-Juan Li, Tao Wang, Yu-Xuan Tao, Mei Zhang, Wen Gu, Jie Yu, Xing-Xin Yang

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Li-Ping Yu, Yan-Juan Li, Tao Wang, Yu-Xuan Tao, Mei Zhang, Wen Gu, Jie Yu, Xing-Xin Yang, College of Pharmaceutical Science, Yunnan University of Chinese Medicine, Kunming 650500, Yunnan Province, China

Li-Ping Yu, Yan-Juan Li, Tao Wang, Yu-Xuan Tao, Mei Zhang, Wen Gu, Jie Yu, Xing-Xin Yang, College of Pharmaceutical Science, Yunnan Key Laboratory of Southern Medicine Utilization, Kunming 650500, Yunnan Province, China

Corresponding author: Xing-Xin Yang, PharmD, Professor, College of Pharmaceutical Science, Yunnan University of Chinese Medicine, No. 1076 Yuhua Road, Kunming 650500, Yunnan Province, China. yxx78945@163.com

Abstract

BACKGROUND

Metabolic dysfunction-associated fatty liver disease (MAFLD) is a severe threat to human health. *Polygonum multiflorum* (PM) has been proven to remedy mitochondria and relieve MAFLD, but the main pharmacodynamic ingredients for mitigating MAFLD remain unclear.

AIM

To research the active ingredients of PM adjusting mitochondria to relieve high-fat diet (HFD)-induced MAFLD in rats.

METHODS

Fat emulsion-induced L02 adipocyte model and HFD-induced MAFLD rat model were used to investigate the anti-MAFLD ability of PM and explore their action mechanisms. The adipocyte model was also applied to evaluate the activities of PM-derived constituents in liver mitochondria from HFD-fed rats (mitochondrial pharmacology). PM-derived constituents in liver mitochondria were confirmed by ultra-high-performance liquid chromatography/mass spectrometry (mitochondrial pharmacochemistry). The abilities of PM-derived monomer and monomer groups were evaluated by the adipocyte model and MAFLD mouse model, respectively.

RESULTS

PM repaired mitochondrial ultrastructure and prevented oxidative stress and

energy production disorder of liver mitochondria to mitigate fat emulsion-induced cellular steatosis and HFD-induced MAFLD. PM-derived constituents that entered the liver mitochondria inhibited oxidative stress damage and improved energy production against cellular steatosis. Eight chemicals were found in the liver mitochondria of PM-administrated rats. The anti-steatosis ability of one monomer and the anti-MAFLD activity of the monomer group were validated.

CONCLUSION

PM restored mitochondrial structure and function and alleviated MAFLD, which may be associated with the remedy of oxidative stress and energy production. The identified eight chemicals may be the main bioactive ingredients in PM that adjusted mitochondria to prevent MAFLD. Thus, PM provides a new approach to prevent MAFLD-related mitochondrial dysfunction. Mitochondrial pharmacology and pharmacochemistry further showed efficient strategies for determining the bioactive ingredients of Chinese medicines that adjust mitochondria to prevent diseases.

Key Words: Fatty liver; Mitochondria; Pharmacodynamic ingredients; *Polygonum multiflorum*

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Core Tip: We found that *Polygonum multiflorum* (PM) protected the mitochondrial ultrastructure and prevented oxidative stress and energy production disorder in the liver mitochondria to mitigate metabolic dysfunction-associated fatty liver disease (MAFLD). Eight chemicals were identified from the liver mitochondria of the PM-treated rats using a novel strategy based on mitochondrial pharmacology and pharmacochemistry. The constituents identified regulated mitochondria to alleviate MAFLD. Our results indicate that PM restored mitochondrial structure and function and alleviated MAFLD, which may be related to oxidative stress and energy production. The eight substances may be the main pharmacodynamic ingredients in PM that regulate mitochondria to prevent MAFLD.

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INTRODUCTION

Metabolic dysfunction-associated fatty liver disease (MAFLD) was considered a better definition of nonalcoholic fatty liver disease. In addition to causing cirrhosis and hepatocellular carcinoma, MAFLD is also closely related to the occurrence of a range of metabolic diseases. MAFLD affects the health of 30% of adults worldwide and represents a heavy clinical and economic burden on patients and society [1]. To date, no pharmacotherapy targeting MAFLD is available, except for preventative lifestyle improvements and physical exercise to lose weight[2,3]. Thus, it is urgent to find a cure for this complex liver disease[4].

The study found that mitochondrial dysfunction plays a significant role in the development of MAFLD. The oxidative damage of mitochondrial fatty acids and the decline of mitochondrial quality are considered to be extremely important motivations in the development of MAFLD[5]. Thus, modulation of mitochondrial function may be an effective strategy to alleviate MAFLD.

Polygonum multiflorum (PM) has been used as a traditional medicine for many centuries in China. Its main chemical components include anthraquinones, flavonoids, phenols, and phospholipids. Recent studies have shown that PM has many pharmacological activities, including hepatoprotective, antihyperlipidemic, anti-inflammatory, antioxidant, anticancer, immunomodulatory, and neuroprotective effects[6]. PM extracts can remedy mitochondrial dysfunction in the liver to alleviate MAFLD, such as the promotion of β-oxidation of fatty acids and the activity of carnitine palmitoyltransferase 1[7,8]. In addition, PM regulated lipid metabolism by reducing very low-density lipoprotein and diacylglycerol acyltransferase levels, downregulating hydroxyl methyl-glutaryl coenzyme A reductase, and upregulating cholesterol 7-alpha-hydroxylase or cytochrome P450 7A[9]. Although PM shows treatment efficacy against MAFLD through regulating mitochondria, the bioactive substances involved are still unclear, which acutely limits the development and utilization of PM.

In our previous study, a new strategy of mitochondrial pharmacology and pharmacochemistry for the investigation of pharmacodynamic substances in traditional Chinese medicines (TCMs) was proposed. Using this strategy, the main active ingredients in PM that adjust mitochondria to relieve MAFLD were identified. The results indicated that this strategy could be applied for the efficient search for *in vivo* mitochondria-regulated constituents in TCMs[10].

Here, we investigated the bioactive substances of PM extracts remedying mitochondria to alleviate MAFLD induced by a high-fat diet (HFD) through mitochondrial pharmacology and pharmacochemistry, after determining the efficacy and potential principle of PM on MAFLD and mitochondria *in vitro* and *in vivo* pharmacodynamic tests. The results showed that PM relieved HFD-induced MAFLD by improving oxidative stress and energy production. The five substances recognized by the proposed method may be the main active ingredients in PM that adjust mitochondria to relieve MAFLD. Thus, PM represents a promising drug for the treatment of MAFLD-related mitochondrial dysfunction to relieve MAFLD. Additionally, mitochondrial pharmacology and pharmacochemistry were efficient strategies for identifying the bioactive ingredients of TCMs regulating mitochondria to prevent human disorders.

MATERIALS AND METHODS

Reagents and chemicals

Standards including resveratrol [(RES), ≥ 98%], emodin [(ED), ≥ 98%], aloe-emodin [(AE), ≥ 98%], emodin 8-O-glucoside [(EG), ≥ 98%], 2,3,5,4′-tetrahydroxy stilbene 2-O-β-D-glucoside [(THSG), ≥ 98%], and physcion [(PN), ≥ 98%] were obtained from Chengdu Pufei De Biotech Co., Ltd. (Chengdu, China). Torachrysone [(TCS), ≥ 98%] was purchased from Yunnan Xili biology science and technology co., ltd (Kunming, China). The dried root of PM was collected from the Luoshiwan Chinese Medicine Market (Kunming, China). Dried PM decoction pieces were verified by Professor Jie Yu. PM credential specimens (No. 19080322) were stored in the Key Laboratory of Southern Medicine Utilization, Yunnan University of Chinese Medicine (Kunming, China). The other chemicals and reagents used were described in our previous report[10].

PM extract preparation

After PM was processed according to the method specified in the 2020 Chinese Pharmacopoeia and decocted with 10 times the amount of 90% ethanol for 110 min. The filtrate was collected after filtration. Dregs were decocted with 10 times 50% ethanol for 110 min and filtered. Then, two filtrates were mixed and condensed at 65 °C. Finally, the concentrate was freeze-dried into a powder.

The chemical characteristics of PM extract were assayed according to the regulation described in the 2020 Chinese Pharmacopoeia. PM was assayed by Shimadzu LC-30A ultra-high performance liquid chromatography (UHPLC). Analytical conditions were as follows: (1) Shimadzu UHPLC column (Shim-pack XR-ODS C8, 100 mm × 2.0 mm I.D., 2.2 μm); (2) Mobile phase for the separation of ED and PN: methanol and 0.1% aqueous phosphoric acid solution (80:20 v/v); (3) Mobile phase for the separation of THSG: Acetonitrile and water solution (25:75 v/v); (4) Detection wavelength: 254 nm for ED and PN; 320 nm for THSG; (5) Temperature: 30 °C; (6) Flow rate: 0.2 mL/min; and (7) Volume: 10 μL. References of ED, PN, and THSG were dissolved in methanol to yield contents of 0.08 mg/mL, 0.04 mg/mL, and 0.2 mg/mL, respectively. For the detection of THSG and for the detection of ED and PN, 0.1 g and 0.02 g, respectively, of PM powder was dissolved in 1.0 mL of methanol. Concentrations of free anthraquinone (ED and PN) and THSG in the PM extract were 0.57% and 0.71%, respectively.

In vitro assay of anti-steatosis activity of PM extract

L02 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C and 5% CO_2. After growing to about 80%, the cells were treated with lipid emulsion. Then, the cells were stimulated by PM extracts (10, 50, and 100 μg/mL) and fenofibrate capsules (150 μmol/L) or a media (control) for 24 h. Bicinchoninic acid (BCA) assay was used to analyze protein concentrations in cell lysates. Triglyceride (TG), total cholesterol (TC), aspartate transaminase (AST), alanine transaminase (ALT), superoxide dismutase (SOD), glutathione (GSH), ATP synthase (ATPase), complex I, and complex II levels were determined using the kits. All data were obtained by the microplate reader. Furthermore, for the detection of intracellular neutral lipid accumulation, L02 cells were seeded into 6-well chambers. The collected cells were washed twice with ice-cold phosphate-buffered saline and fixed with 2.5% paraformaldehyde for 30 min. Afterward, the cells were added with 60% isopropanol and cultured for 5 min. Then, cells were stained with 0.2% Oil red O solution for 30 min. After washing twice with phosphate-buffered saline, cells were stained with hematoxylin for 3 min. Finally, images were obtained using a CX31 Olympus Imaging System.

In vivo determination of PM extract against MAFLD

Experimental animals: Healthy male Sprague Dawley rats (200 ± 50 g) were provided by Dashuo

Biotech. Co., Ltd. (Chengdu, China). All animals were raised in conditions of 22 ± 1 °C, a humidity of 60% ± 10%, and a light/dark cycle of 12 h with free access to food and water. The experiments in this study were approved by the Institutional Ethical Committee on Animal Care and Experimentations of Yunnan University of Chinese Medicine (R-06201965).

Grouping and administration of treatment: After 5-7 d of adaptive feeding, the rats were randomly divided into six groups (n = 6): (1) Normal control group (NC); (2) Model group (MOD); (3) RES group (40 mg/kg); (4) Low-dose PM group (2 g/kg); (5) Middle-dose PM group (4 g/kg); and (6) High-dose PM group (8 g/kg). Rats were given the test samples by intragastric administration once a day for 3 mo. Except for the NC group, rats in all groups were fed an HFD (10% egg yolk, 10% refined lard, 79% basic diet, and 1% cholesterol) to induce MAFLD in rats for 3 mo.

Sample collection: The daily food intake of all rats was recorded, and weight was recorded once a week. At the end of the experiment, the rats were anesthetized by intraperitoneal injection with 10% sodium pentobarbital. Blood samples were obtained from the abdominal aorta and coagulated at 4 °C. Blood was then centrifuged at 3500 rpm for 15 min to acquire serum. The liver, kidneys, and spleen were weighed. A portion of the liver was applied for Oil red O staining and transmission electron microscopy examination as previously described[10], and the remaining portion was stored at -80 °C.

Measurement of biochemical indicators in the serum and liver: Serum levels of TC, TG, low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), ALT, and AST were determined following the kit instructions. In addition, liver homogenates were prepared with nine volumes of normal saline and were centrifuged at 2000 r/min for 10 min. The levels of TC, TG, LDL-C, HDL-C, ALT, and AST in the liver supernatant were detected according to the instructions of the kits. BCA assay was used to determine protein concentration in the liver. All data were collected by a microplate reader.

Detection of liver mitochondrial indices: Liver mitochondria were obtained as in previous studies[11] and resuspended in saline. BCA assay was used to determine protein concentration. The levels of malondialdehyde (MDA), SOD, GSH, ATPase, and complex I and II in the suspension of mitochondria were detected according to the instructions of the kit. All data were collected by a microplate reader.

Activity evaluation and structural assignment of PM-derived constituents in mitochondria from HFD-induced rats

Production of liver mitochondrial extracts: The mitochondria of rat livers in the MOD group and HPM group were separated as previously reported[11]. The obtained mitochondria were suspended in chromatographic methanol and then ultrasonicated for 20 min followed by centrifugation at 10000 × g at 4 °C for 25 min. The supernatant was collected and dried with mild nitrogen, and mitochondrial extracts were stored at -80 °C.

***In vitro* assay of anti-steatosis ability of PM-derived constituents in mitochondria:** L02 cells were stimulated with 5% fat emulsion for 24 h and then cultured with fenofibrate capsules (150 μmol/L) and mitochondrial extracts (50, 100, and 200 μg/mL) of rats in the MOD group and HPM group for 24 h. Cells were collected after being washed twice with phosphate-buffered saline. BCA assay was used to determine the protein concentration in the cell lysate. TC, TG, SOD, GSH, complex I, complex II, and ATPase levels in the cell were detected according to the instructions of the kits. All data were collected by a microplate reader. Additionally, cells stained with Oil red O were examined as in the aforementioned method.

Structural assignment of PM-derived constituents in liver mitochondria: PM extracts and liver mitochondrial extracts isolated from the MOD and HPM groups were redissolved in HPLC-grade methanol and filtered through a 0.22 μm Millipore filter (Bedford, MA, United States). The filtrate was analyzed by a UHPLC Dionex Ultimate 3000 system coupled with a Thermo Scientific Q-Exactive TM hybrid quadrupole-orbitrap mass spectrometry (MS) (Thermo Fisher Scientific, San Jose, CA, United States). The detailed analytical conditions of UHPLC and MS were described in our previous report[10].

***In vitro* assay of anti-steatosis ability of PM-derived monomer in mitochondria:** The anti-steatosis activity of TCS, a PM-derived monomer, on L02 cells induced by fat emulsion was measured. First, the cell activity of TCS at different concentrations (0, 6.25, 12.5, 25, 50, 100, and 200 μmol/L) was measured according to the CCK-8 kit's instructions. Subsequently, fat emulsion-treated steatosis cells were introduced with TCS (12.5 and 25 μmol/L). BCA assay was used to analyze the protein concentration of the cell lysate. The levels of TG, TC, AST, ALT, SOD, GSH, and ATPase were measured on the microplate reader according to the instructions of the kits.

***In vivo* determination of anti-MAFLD activity of the PM-derived monomer group in liver mitochondria:** Effects of the PM-derived monomer group on MAFLD were evaluated on HFD-induced mice. The monomer group consisted of ED, AE, EG, and THSG in a ratio of 14.87:1:10.03:18.37. This ratio was the concentration ratio of these molecules in the PM extract counted according to the areas of the

chromatographic peak obtained in the UHPLC/MS assay of PM. Male C57BL/6J mice (20 ± 2 g) were provided from Hunan SJA Laboratory Animal Co., Ltd. (Hunan, China). After 5-7 d of adaptive feeding, mice were fed with a normal chow diet as the control group (n = 6), and other groups were fed an HFD for 8 wk to induce MAFLD. Mice in the treatment groups (n = 6) were intraperitoneally injected as follows: (1) A low-dose PM-derived monomer group in the mitochondria (30 mg/kg/d); (2) High-dose PM-derived monomer group in the mitochondria (90 mg/kg/d); and (3) RES (18 mg/kg/d). MAFLD was induced by an HFD for 3 mo. Starting from the 9th wk, mice received an intraperitoneal injection with the test samples once a day for 4 wk. The methods of sample collection and serum, liver, and mitochondrial indices detection were the same as the experiment of PC extract administered to rats described above.

Statistical analysis

All data were represented as the mean ± SD. Statistical analysis was performed on GraphPad Prism version 8.0 software for Windows (San Diego, CA, United States). One-way analysis of variance was used for comparison between groups. Statistical significance is displayed as $P < 0.05$.

RESULTS

Effects of PM extract on fat emulsion triggered L02 adipocytes

The inhibitory activity of PM extracts was assessed by 5% fat emulsion-induced intracellular lipid accumulation in L02 cells. Oil red O staining revealed that fat emulsion-treated cells had higher intracellular lipid levels compared to untreated L02 cells (NC). However, treatment with PM extracts greatly blocked fat emulsion-induced lipid accumulation (Figure 1A). As shown in Figures 1B-K, after 24 h of stimulation with fat emulsion, TG, TC, AST, and ALT levels remarkably increased, and GSH, SOD, Na^+-K^+-ATPase, Ca^{2+}-Mg^{2+}-ATPase, complex I, and complex II levels declined. However, after PM incubation, ALT and AST levels decreased, and SOD, GSH, Na^+-K^+-ATPase, Ca^{2+}-Mg^{2+}-ATPase, and complex II levels markedly increased. The levels of TC and TG showed a decreasing trend, and the level of complex I showed an increasing trend. Together, these findings suggested that PM extract relieved the steatosis of L02 cells.

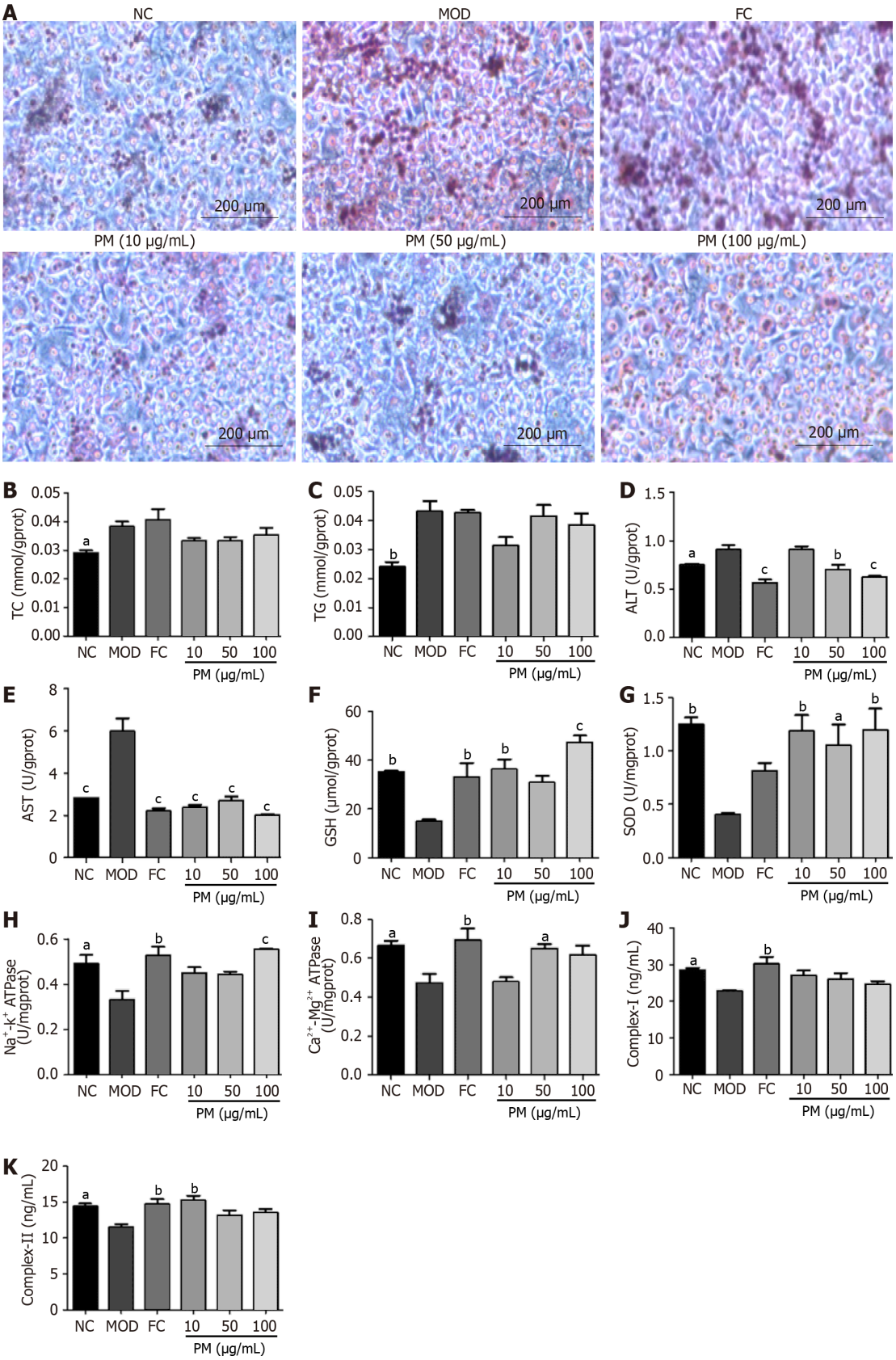
Effects of PM extract on MAFLD rats induced by HFD-feeding

Effect of PM on body weight, food consumption, and organ indices of rats: As shown in Table 1, without affecting food intake, rats fed an HFD for 3 mo gained weight at the end of the study. Moreover, after 3 mo of HFD feeding, the liver index of rats increased remarkably, and body weight, kidney index, and spleen index showed an increasing trend. However, the administration of PM extract significantly decreased the spleen index, and body weight, liver index, and kidney index showed a decreasing trend. Thus, these data suggested that PM relieved HFD-induced weight gain and organ enlargement.

Effects of PM extract on serum parameters: As displayed in Figures 2A-F, after 3 mo of an HFD, ALT levels dramatically increased, while HDL-C levels markedly decreased. Additionally, the levels of TC, TG, AST, and LDL-C showed an increasing trend. After administration with PM extract, TG, ALT, and AST levels decreased obviously, and HDL-C level markedly increased. LDL-C levels showed a decreasing trend. Taken together, these data suggested that PM relieved HFD-induced dyslipidemia.

Effects of PM extract on liver status: Figure 3A shows the massive accumulation of lipid droplets, diffuse hepatocellular edema, and degeneration in the liver of rats after 3 mo of HFD feeding. However, after administration with PM extract and RES, the lipid droplets in the liver cells of rats reduced, and liver status tended to be normal. Additionally, Figures 3B-E shows that the levels of TC and TG improved obviously after 3 mo of HFD feeding. The level of AST showed an increasing trend, and the level of HDL-C showed a decreasing trend. After treatment with PM extract, TG and AST levels dramatically declined, and HDL-C levels dramatically increased. Thus, these findings suggested that PM extract reduced lipid accumulation in the liver of HFD-fed rats.

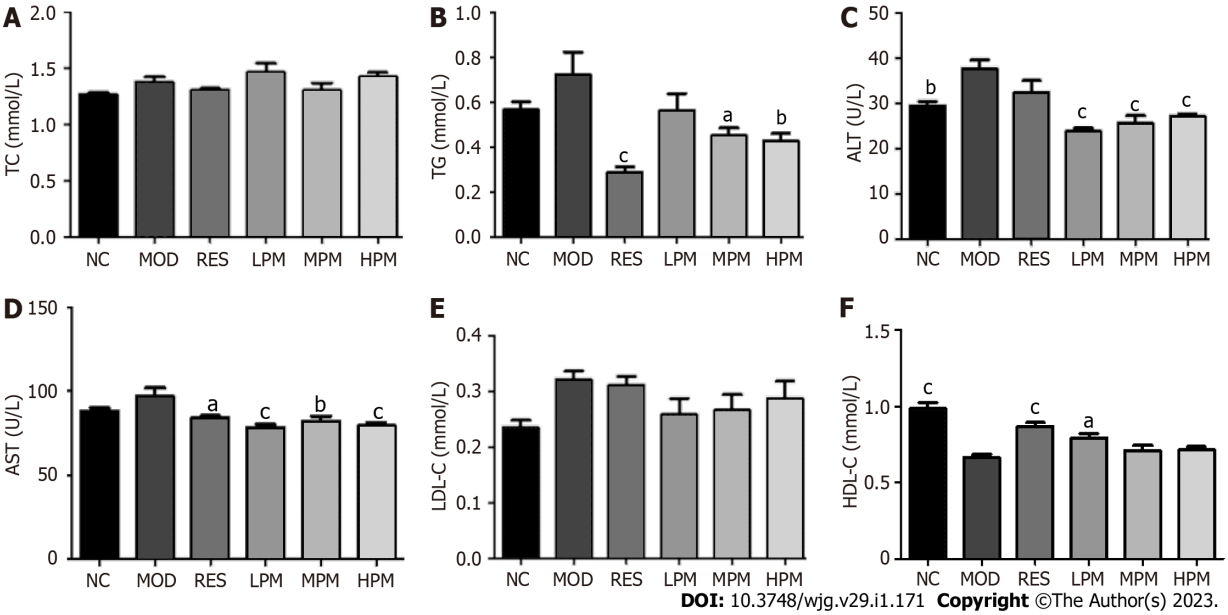
Effects of PM extract on mitochondrial status: Figure 4A shows that after the feeding of an HFD, the liver mitochondria swelled. The inner and outer membranes and ridges were blurred, and the matrix was unclear, as indicated by the red arrow in the MOD group. However, the ultrastructural appearance of mitochondria was reversed by PM treatment. Additionally, Figures 4B-H shows that after the feeding of an HFD, SOD, complex I, and complex II levels in the mitochondria decreased obviously. Furthermore, GSH, Na^+-K^+-ATPase, and Ca^{2+}-Mg^{2+}-ATPase levels in liver mitochondria showed a decreasing trend, and the MDA level in the liver mitochondria showed an increasing trend. After administration with PM extract, SOD, Na^+-K^+-ATPase, Ca^{2+}-Mg^{2+}-ATPase, complex I, and complex II levels remarkably increased, and the level of MDA remarkably decreased. The level of GSH showed an increasing trend. Combined, these indices suggested that PM prevented damage to mitochondrial



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Figure 1 Effects of *Polygonum multiflorum* extract on L02 adipocytes induced by fat emulsion. A: L02 cells were treated with 5% fat emulsion for 24 h followed by *Polygonum multiflorum* extract (10, 50, and 100 μg/mL) or fenofibrate capsules (150 μmol/L) for 24 h. Cells were harvested, and fat accumulation in cells was detected under a light microscope (× 200) after Oil red O staining; B: Levels of total cholesterol was determined using commercial kits; C: Levels of triglyceride was determined using a commercial kit; D: Levels of alanine transaminase were determined using a commercial kit; E: Levels of aspartate transaminase were determined using a commercial kit; F: Levels of glutathione were determined using a commercial kit; G: Levels of superoxide dismutase were determined using

a commercial kit; H: Levels of Na^+-K^+-ATPase were determined using a commercial kit; I: Levels of Ca^{2+}-Mg^{2+}-ATPase were determined using a commercial kit; J: Levels of complex I were determined using a commercial kit; K: Levels of complex II were determined using a commercial kit. Data were obtained from 3 independent measurements and are expressed as the mean ± SD. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$ *vs* model group. ALT: Alanine transaminase; AST: Aspartate transaminase; ATPase: ATP synthase; Complex I/II: human mitochondrial respiratory chain complex I/II, FC: Fenofibrate capsules; GSH: Glutathione; MOD: Model; NC: Normal control; PM: *Polygonum multiflorum*; SOD: Superoxide dismutase; TC: Total cholesterol; TG: Triglyceride.



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Figure 2 Effects of *Polygonum multiflorum* extract on serum biochemical parameters of high-fat diet-fed rats. Sprague Dawley rats were administered saline, resveratrol, or *Polygonum multiflorum* extract (2, 4 or 8 g/kg/d). After 3 mo of high-fat diet feeding, rats were sacrificed, and serum was collected. A: Serum levels of total cholesterol were determined using a commercial kit; B: Serum levels of triglyceride were determined using a commercial kit; C: Serum levels of alanine transaminase were determined using a commercial kit; D: Serum levels of aspartate transaminase were determined using a commercial kit; E: Serum levels of low-density lipoprotein cholesterol were determined using a commercial kit; F: Serum levels of high-density lipoprotein cholesterol were determined using a commercial kit. Values represent the mean ± SD from six animals. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$ *vs* model group. ALT: Alanine transaminase; AST: Aspartate transaminase; HDL-C: High-density lipoprotein cholesterol; HPM: High-dose *Polygonum multiflorum* extract; LDL-C: Low-density lipoprotein cholesterol; LPM: Low dose *Polygonum multiflorum* extract; MOD: Model; MPM: Middle-dose *Polygonum multiflorum* extract; NC: Normal control; RES: Resveratrol; TC: Total cholesterol; TG: Triglyceride.

structure and functions to alleviate MAFLD.

Effects of PM-derived constituents in mitochondria on adipocytes induced by fat emulsion

Oil red O staining revealed that fat emulsion-triggered L02 cells had higher intracellular lipid levels compared to untreated L02 cells (NC). After liver mitochondrial extracts from HPM treatment, intracellular lipid accumulation was prevented and was not prevented by liver mitochondrial extracts from MOD treatment (Figure 5A). As shown in Figures 5B-I, after incubation with fat emulsion, the levels of TC and TG increased, and GSH, SOD, Na^+-K^+-ATPase, Ca^{2+}-Mg^{2+}-ATPase, complex I, and complex II levels decreased. After incubation with liver mitochondrial extracts from HPM, TC levels decreased, and GSH, SOD, Na^+-K^+-ATPase, and Ca^{2+}-Mg^{2+}-ATPase levels increased. Furthermore, the TG level showed a downward trend, and levels of complex I and complex II displayed an increasing trend. However, liver mitochondrial extracts from MOD treatment did not reverse the pathological changes induced by fat emulsion, except for the significantly reversed TC level. These results showed that PM-derived constituents in the liver mitochondrial extracts ameliorated cellular steatosis by regulating oxidative stress and energy production.

Structural assignment of PM-derived constituents in liver mitochondria

After analyzing accurate high-resolution MS_n data provided by UHPLC/MS (Table 2) and comparing the data with antecedently described[12-19] and standards, eight PM-derived monomers, including five prototype monomers and three of their metabolites (Figure 6), were screened in mitochondrial liver extracts of PM-treated rats. Among the identified monomers, there were one stilbene, five anthraquinones, and two naphthols. Prototype monomers consisted of ED, AE, EG, THSG, and TCS. The metabolites comprised emodin-glucuronide, torachrysone-glucuronide, and emodin-8-O-β-D-glucopyranoside reduction metabolite, which originated from ED, THCS, and EG, respectively.

Table 1 Effects of *Polygonum multiflorum* extract on body weight, food intake, and organ indices of high-fat diet-fed rats

	NC	MOD	RES	LPM	MPM	HPM
Initial body weight (g)	263.67 ± 23.43	263.83 ± 28.54	261.50 ± 16.99	275.83 ± 6.05	268.83 ± 12.42	246.00 ± 15.09
Final body weight (g)	458.67 ± 46.43	470.17 ± 89.14	424.67 ± 25.97	454.67 ± 16.98	479.83 ± 33.88	432.83 ± 47.94
Body weight gain (g)	195.00 ± 23.00	206.33 ± 60.60	163.17 ± 8.98	178.83 ± 10.93	211.00 ± 21.46	186.83 ± 32.86
Food intake (g)	15852.54	14023.10	14265.09	13828.25	13081.76	13645.74
Liver index (%)	2.15 ± 0.09[b]	2.64 ± 0.22	2.32 ± 0.12[a]	2.55 ± 0.11	2.49 ± 0.16	2.49 ± 0.20
Kidney index (%)	0.52 ± 0.02	0.54 ± 0.07	0.52 ± 0.02	0.50 ± 0.02	0.49 ± 0.03	0.54 ± 0.04
Spleen index (%)	0.1589 ± 0.01	0.1618 ± 0.02	0.1432 ± 0.01	0.1267 ± 0.01[b]	0.1427 ± 0.01	0.1345 ± 0.01[a]

[a]$P < 0.01$ *vs* model group.
[b]$P < 0.001$ *vs* model group.
Values represent the mean ± SD from six animals. HPM: High-dose *Polygonum multiflorum* extract; LPM: Low dose *Polygonum multiflorum* extract; MOD: Model; MPM: Middle-dose *Polygonum multiflorum* extract; NC: Normal control; RES: Resveratrol.

Table 2 Ultra-high performance liquid chromatography/mass spectrometry data and chemical structure assignment of *Polygonum multiflorum*-derived monomers from liver mitochondria

No	T_R (min)	(M-H)- (m/z)	Molecular formula	Predicted (m/z)	Measured (m/z)	Diff (ppm)	ESI-MSn(-), m/z (abundance)	Assigned identification	Ref.
1	18.87	269.0454	$C_{15}H_{10}O_5$	269.0450	269.0454	3.457	269.04541 (100.00), 241.05089 (2.18), 225.14899 (1.98)	Aloe-emodin[1]	[12-14]
2	15.90	431.0981	$C_{21}H_{20}O_{10}$	431.0978	431.0981	2.011	431.109811 (34.23), 311.05627 (4.32), 269.04553 (100.00), 240.04503 (0.69)	Emodin-8-O-glucoside[1]	[14-16]
3	33.91	269.0457	$C_{15}H_{10}O_5$	269.0450	269.0457	4.461	269.04553 (100), 241.05331 (0.18), 225.05482 (1.37)	Emodin[1]	[15-19]
4	18.80	445.0778	$C_{21}H_{18}O_{11}$	445.0771	445.0778	2.881	269.04553 (100.00), 225.05527 (0.19)	Emodin-glucuronide	[17]
5	9.06	405.1190	$C_{20}H_{22}O_9$	405.1186	405.1190	2.422	243.06613 (62.27)	2,3,5,4′-Tetrahydroxy stilbene-[1]	[17]
6	13.17	433.1136	$C_{21}H_{22}O_{10}$	433.1135	433.1136	1.539	257.08191 (100.00), 175.02435 (8.79), 152.99582 (3.58), 113.02423 (59.47)	Emodin-8-O-β-D-glucopyranoside reduction metabolite	[17]
7	13.46	245.0820	$C_{14}H_{14}O_4$	245.0814	245.0820	4.834	245.08208 (87.57), 230.05850 (9.07)	Torachrysone[1]	[17]
8	15.15	421.1142	$C_{20}H_{22}O_{10}$	421.1135	421.1142	3.032	245.08179 (76.39), 230.05852 (4.02)	Torachrysone-glucuronide	[17]

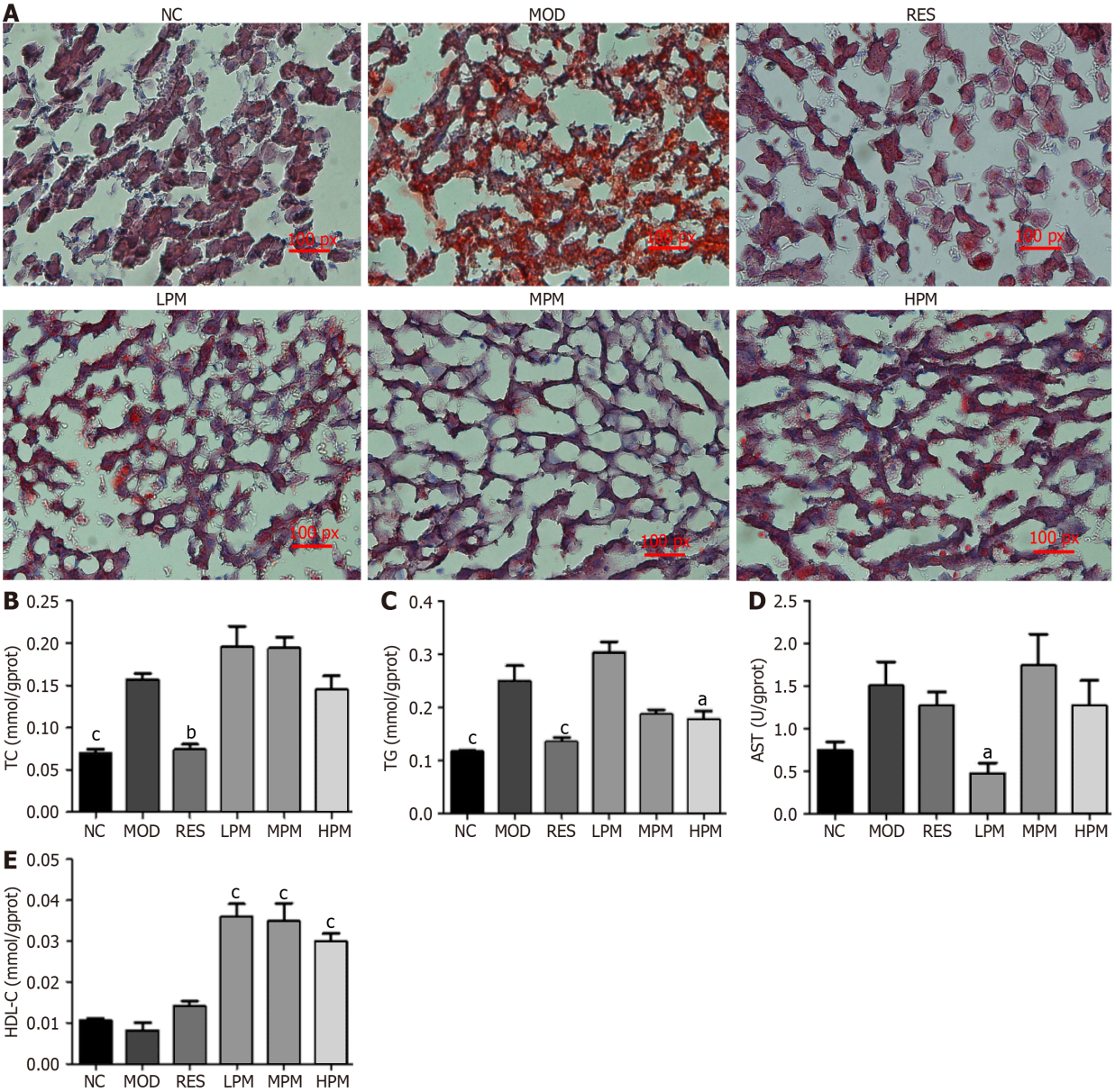
[1]Compared with reference substances.

Effects of PM-derived monomer in mitochondria of steatosis cells

As shown in Figure 7, after incubation with fat emulsion, the levels of TC, TG, ALT, and AST dramatically increased, and GSH, SOD, Na^+-K^+-ATPase, and Ca^{2+}-Mg^{2+}-ATPase levels dramatically decreased. After TCS stimulation, TC and AST levels decreased, and the levels of GSH, SOD, and Ca^{2+}-Mg^{2+}-ATPase increased. The levels of TG and ALT showed a decreasing trend. These data suggested that TCS mitigated fat emulsion-triggered cellular steatosis.

Effects of PM-derived monomer group in mitochondria on MAFLD mice induced by HFD-feeding

Effects of PM-derived monomer group on body weight, food consumption, and organ index of MAFLD mice: As shown in Table 3, without affecting food intake, mice fed an HFD for 3 mo gained weight at the end of the study. The liver and kidney index increased obviously, and the spleen index showed an increasing trend. After treatment with the PM-derived monomer group, the liver index decreased. Body weight and the kidney index showed a decreasing trend, whereas the spleen index level increased in the HMG group. Thus, these data indicated that the PM-derived monomer group reduced weight gain and organ index swelling caused by HFD.

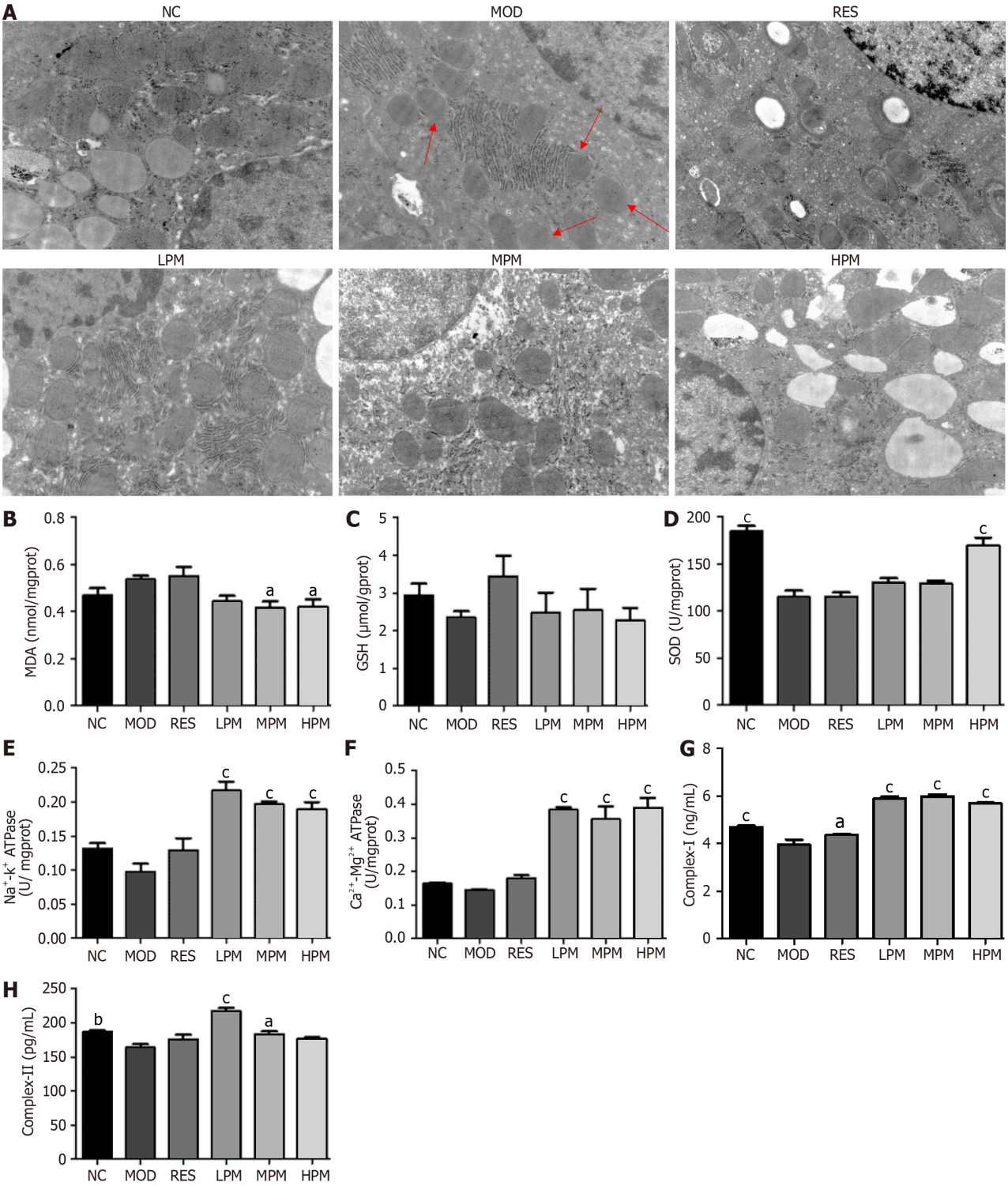


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Figure 3 Effects of *Polygonum multiflorum* extract on the liver lipid content of high-fat diet-fed rats. Sprague Dawley rats were administered saline, resveratrol, or *Polygonum multiflorum* extract (2, 4, or 8 g/kg/d). After 3 mo of high-fat diet feeding, rats were sacrificed, and livers were harvested. A: Liver histology after Oil Red O staining was observed under a light microscope (× 200); B: Liver levels of total cholesterol were determined using a commercial kit; C: Liver levels of triglyceride were determined using a commercial kit; D: Liver levels of aspartate transaminase were determined using a commercial kit; E: Liver levels of high-density lipoprotein cholesterol were determined using a commercial kit. Values represent the mean ± SD from six animals. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* the relevant model group. AST: Aspartate transaminase; HDL-C: High-density lipoprotein cholesterol; HPM: High-dose *Polygonum multiflorum* extract; LPM: Low dose *Polygonum multiflorum* extract; MOD: Model; MPM: Middle-dose *Polygonum multiflorum* extract; NC: Normal control; RES: Resveratrol; TC: Total cholesterol; TG: Triglyceride.

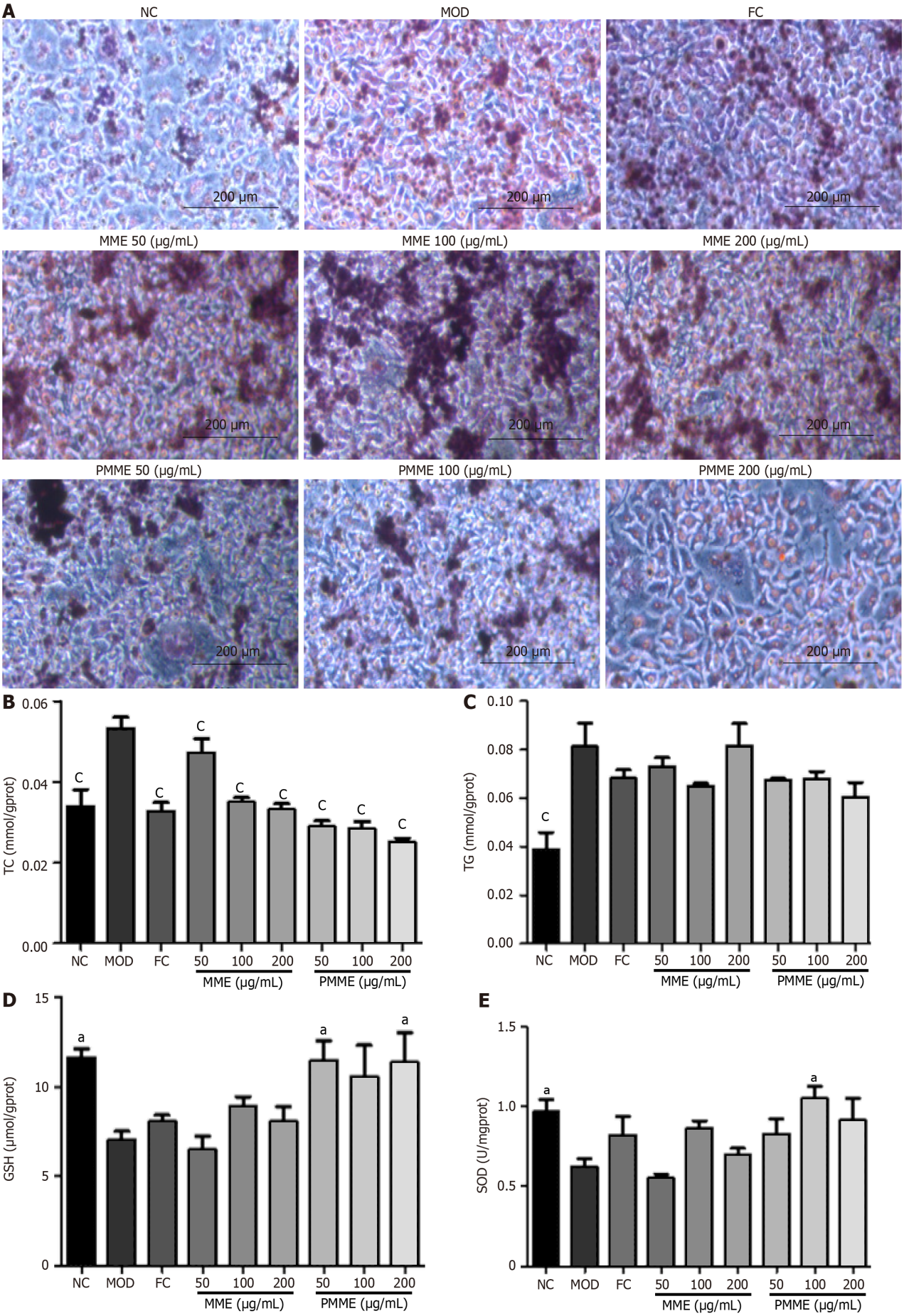
Effects of the PM-derived monomer group on serum indicators: Figures 8A-C show that after 3 mo of HFD feeding, TC and LDL-C levels increased obviously, and the level of AST showed an increasing trend. After treatment with the PM-derived monomer group, the levels of TC, AST, and LDL-C were decreased. Thus, the results show that the PM-derived monomer group alleviated HFD-induced dyslipidemia.

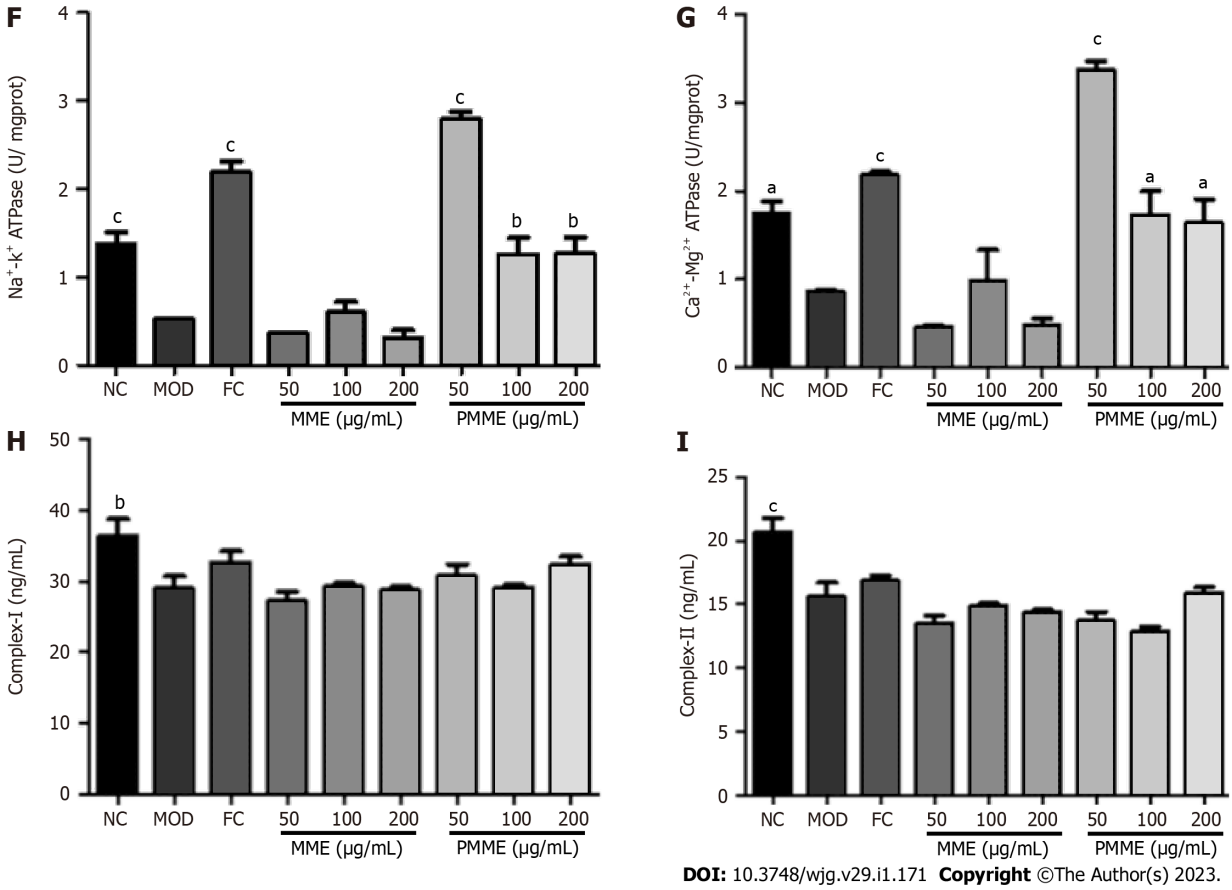
Effects of the PM-derived monomer group on the liver lipid content: As displayed in Figure 9A, after 3 mo of HFD feeding, the livers exhibited lipid accumulation, diffuse hepatocellular edema, and degeneration. However, this status of the livers was restored to normal after administration with PM-derived monomer groups. Figures 9B and 9D shows that after 3 mo of HFD consumption, ALT and AST levels increased, and the level of TC showed an increasing trend. After administration with the PM-derived monomer group, levels of TC, ALT, and AST decreased obviously. These data suggested that the PM-derived monomer group reduced lipid accumulation in the livers of HFD mice.



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Figure 4 Effects of *Polygonum multiflorum* extract on the mitochondrial status of the liver from high-fat diet-fed rats. Sprague Dawley rats were administered saline, resveratrol, or *Polygonum multiflorum* extract (2, 4, or 8 g/kg/d). After 3 mo of high-fat diet feeding, rats were sacrificed, and livers were harvested for observation of the mitochondrial ultrastructure and mitochondrial isolations. A: Mitochondria were assessed by transmission electron microscope (original magnification × 30000; bar = 500 nm); B: Levels of malondialdehyde were determined using a commercial kit; C: Levels of glutathione were determined using a commercial kit; D: Levels of superoxide dismutase were determined using a commercial kit; E: Levels of Na^+-K^+-ATPase were determined using a commercial kit; F: Levels of Ca^{2+}-Mg^{2+}-ATPase were determined using a commercial kit; G: Levels of complex I were determined using a commercial kit; H: Levels of complex II in isolated liver mitochondria were determined using a commercial kit. Values represent the mean ± SD from six animals. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$ *vs* model group. ATPase: ATP synthase; Complex I/II: rat mitochondrial respiratory chain complex I/II, GSH: Glutathione; HPM: High-dose *Polygonum multiflorum* extract; LPM: Low-dose *Polygonum multiflorum* extract; MDA: Malondialdehyde; MOD: Model; MPM: Middle-dose *Polygonum multiflorum* extract; NC: Normal control; RES: Resveratrol; SOD: Superoxide dismutase.


A
NC
MOD
FC
MME 50 (μg/mL)
MME 100 (μg/mL)
MME 200 (μg/mL)
PMME 50 (μg/mL)
PMME 100 (μg/mL)
PMME 200 (μg/mL)
200 μm
B
TC (mmol/gprot)
C
NC MOD FC 50 100 200 50 100 200
MME (μg/mL)
PMME (μg/mL)
C
TG (mmol/gprot)
D
GSH (μmol/gprot)
a
E
SOD (U/mgprot)



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Figure 5 Effects of *Polygonum multiflorum*-derived constituents in liver mitochondria on L02 adipocytes induced by fat emulsion. L02 cells were induced by fat emulsion for 24 h, then treated with liver mitochondrial extracts of the model group (50, 100, and 200 μg/mL), liver mitochondrial extract of high-dose of *Polygonum multiflorum* extract (50, 100, and 200 μg/mL), or fenofibrate capsules (150 μmol/L) for 24 h. Cells were harvested. A: Fat accumulation in cells was evaluated under a light microscope (× 200) after Oil Red O staining; B: Cell levels of total cholesterol were determined using a commercial kit; C: Cell levels of triglyceride were determined using a commercial kit; D: Cell levels of glutathione were determined using a commercial kit; E: Cell levels of superoxide dismutase were determined using a commercial kit; F: Cell levels of Na^+-K^+-ATPase were determined using a commercial kit; G: Cell levels of Ca^{2+}-Mg^{2+}-ATPase were determined using a commercial kit; H: Cell levels of complex I were determined using a commercial kit; I: Cell levels of complex II were determined using a commercial kit. Data were obtained from 3 independent measurements and are expressed as the mean ± SD. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$ *vs* model group. ATPase: ATP synthase; Complex I/II: human mitochondrial respiratory chain complex I/II, FC: Fenofibrate capsules; GSH: Glutathione; MOD: Model; MME: Liver mitochondrial extracts from the model group; NC: Normal control; PMME: Liver mitochondrial extracts from the high-dose of *Polygonum multiflorum* extract group; SOD: Superoxide dismutase; TC: Total cholesterol; TG: Triglyceride.

Effects of PM-derived monomer group on the status of liver mitochondria: As shown in Figure 10A, after 3 mo of HFD feeding, the liver mitochondria swelled. The inner and outer membranes and ridges were blurred, and the matrix was unclear, as indicated by the red arrows in the MOD group. However, treatment with the PM-derived monomer group markedly improved the ultrastructure of mitochondria. Figures 10B and 10C displays that after 3 mo of HFD feeding, Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase levels decreased obviously. However, after treatment with the PM-derived monomer group, the levels of Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase increased. The results showed that the PM-derived monomer group regulated the state of mitochondria to alleviate MAFLD.

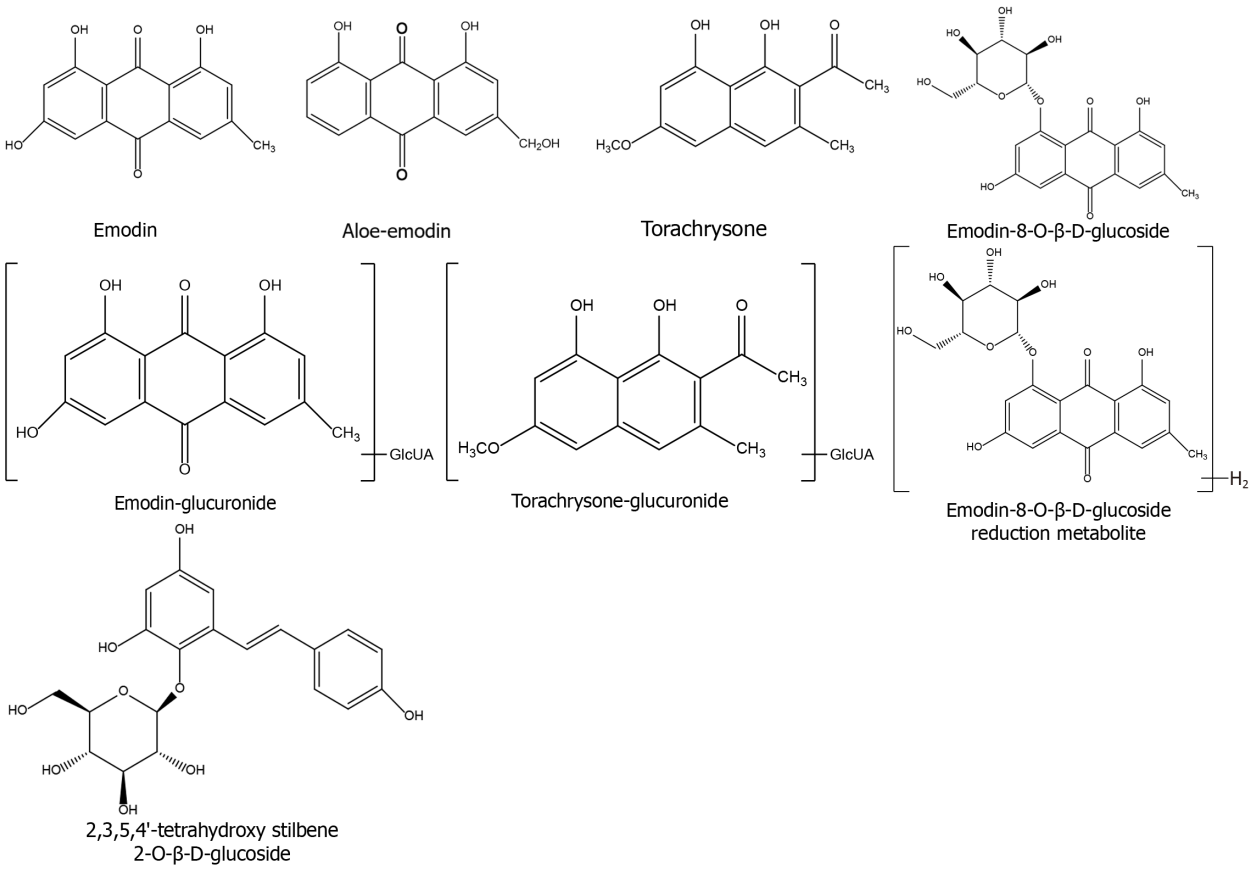
DISCUSSION

In this study, fat emulsion-induced L02 adipocytes and HFD-induced MAFLD rats were established to evaluate the efficacy and underlying mechanism of action of PM on MAFLD and mitochondria. MAFLD primarily manifests as excessive liver fat accumulation, increased serum and liver TC, TG, ALT, AST, LDL-C levels, and decreased HDL-C level[20]. TC, TG, ALT, and AST in L02 cell levels were markedly increased by the induction of fat emulsion, which could be alleviated by PM extracts. Our data also showed that the contents of TC, TG, ALT, AST, and LDL-C in the serum and liver of rats fed with HFD increased, while the level of HDL-C decreased, which could be recovered after PM treatment. Moreover, significant lipid droplet accumulation was observed in the liver of rats fed an HFD, which could be recovered after PM treatment. Together, these findings indicated that PM effectively prevented lipid

Table 3 Effects of the *Polygonum multiflorum*-derived monomer group in liver mitochondria extract on body weight, food intake, and organ indices of rats

	NC	MOD	RES	LMG	HMG
Initial body weight (g)	20.03 ± 0.54	20.38 ± 0.32	20.40 ± 0.59	19.83 ± 0.92	20.33 ± 1.00
Final body weight (g)	22.90 ± 0.56[b]	25.68 ± 1.12	24.22 ± 1.29	25.20 ± 1.74	24.37 ± 1.50
Body weight gain (g)	2.87 ± 0.02	5.30 ± 0.79	3.82 ± 0.70	5.37 ± 0.82	4.03 ± 0.50
Food intake (g)	2274.20	2268.50	2655.80	2473.30	2290.60
Liver index (%)	3.52 ± 0.05[c]	4.24 ± 0.10	3.17 ± 0.11[c]	3.16 ± 0.11[c]	3.50 ± 0.21[c]
Kidney index (%)	1.00 ± 0.57[c]	1.20 ± 0.05	1.11 ± 0.08	1.15 ± 0.08	1.15 ± 0.08
Spleen index (%)	0.21 ± 0.03	0.31 ± 0.06	0.31 ± 0.04	0.30 ± 0.06	0.48 ± 0.22[a]

[a]$P < 0.05$ *vs* model group.
[b]$P < 0.01$ *vs* model group.
[c]$P < 0.001$ *vs* model group.
Values represent the mean ± SD from six animals. HMG: High-dose *Polygonum multiflorum*-derived monomer group; LMG: Low-dose *Polygonum multiflorum*-derived monomer group; MOD: Model; NC: Normal control; RES: Resveratrol.

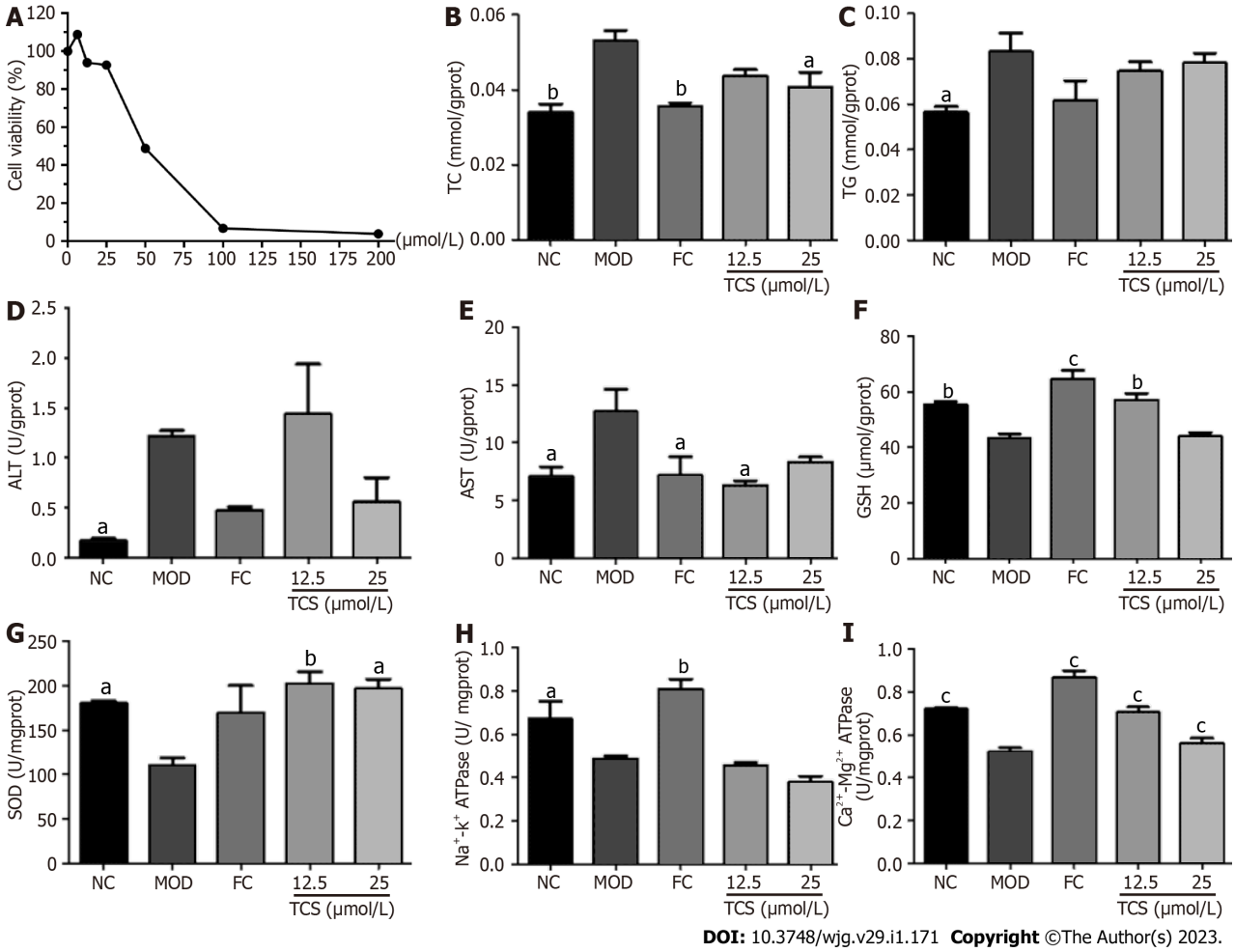


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Figure 6 Chemical structures of *Polygonum multiflorum*-derived constituents from the liver mitochondria extract. GlcUA: Glucuronidation.

metabolism disorders in MAFLD.

Mitochondria regulate a range of important physiological and biochemical cellular processes, such as energy production, apoptosis, oxidative stress, lipid metabolism, and calcium homeostasis. Multiple studies have shown evidence of molecular, biochemical, and biophysical mitochondrial abnormalities in MAFLD initiation and progression[21]. In this study, fat emulsion decreased GSH, SOD, Ca^{2+}-Mg^{2+}-ATPase, Na^{+}-K^{+}-ATPase, complex I, and complex II levels in hepatic cells, which could be recovered to a certain extent by PM treatment. Similarly, the mitochondria of MAFLD rats fed an HFD were swollen and damaged. The levels of SOD, GSH, ATPase, and complex I and II in liver mitochondria of MAFLD

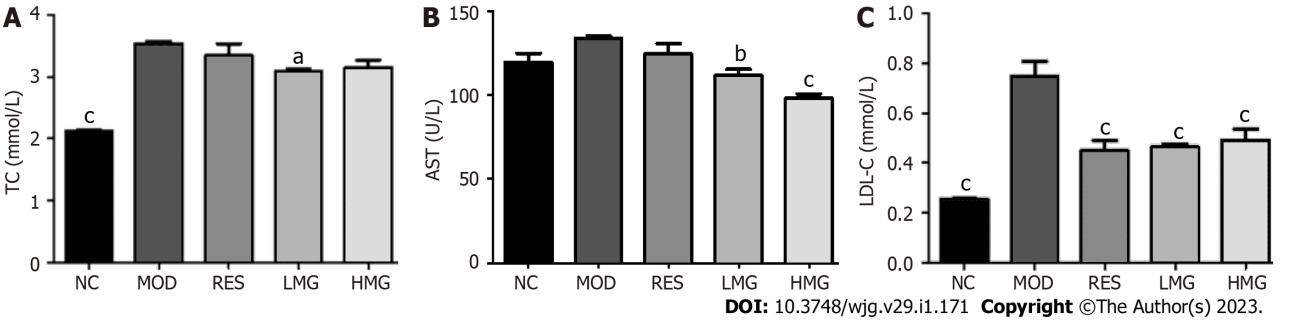


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Figure 7 Effects of *Polygonum multiflorum*-derived monomer in liver mitochondria on L02 adipocytes induced by fat emulsion. A: The cell counting kit-8 assay showed that torachrysone had no obvious cytotoxic effect on L02 cells at 12.5 and 25 μmol/L. These two concentrations were selected for *in vitro* anti-lipid activity detection. L02 cells were induced by fat emulsion for 24 h followed by stimulation with torachrysone (12.5 and 25 μmol/L) or fenofibrate capsules (150 μmol/L) for 24 h. Cells were harvested; B: Levels of total cholesterol were determined using a commercial kit; C: Levels of triglyceride were determined using a commercial kit; D: Levels of alanine transaminase were determined using a commercial kit; E: Levels of aspartate transaminase were determined using a commercial kit; F: Levels of glutathione were determined using a commercial kit; G: Levels of superoxide dismutase were determined using a commercial kit; H: Levels of Na^+-K^+-ATPase were determined using a commercial kit; I: Levels of Ca^{2+}-Mg^{2+}-ATPase were determined using a commercial kit. Data were obtained from 3 independent measurements and are expressed as the mean ± SD. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$ *vs* model group. ALT: Alanine transaminase; AST: Aspartate transaminase; ATPase: ATP synthase; FC: Fenofibrate capsules; GSH: Glutathione; MOD: Model; NC: Normal control; SOD: Superoxide dismutase; TC: Total cholesterol; TG: Triglyceride; TCS: Torachrysone.

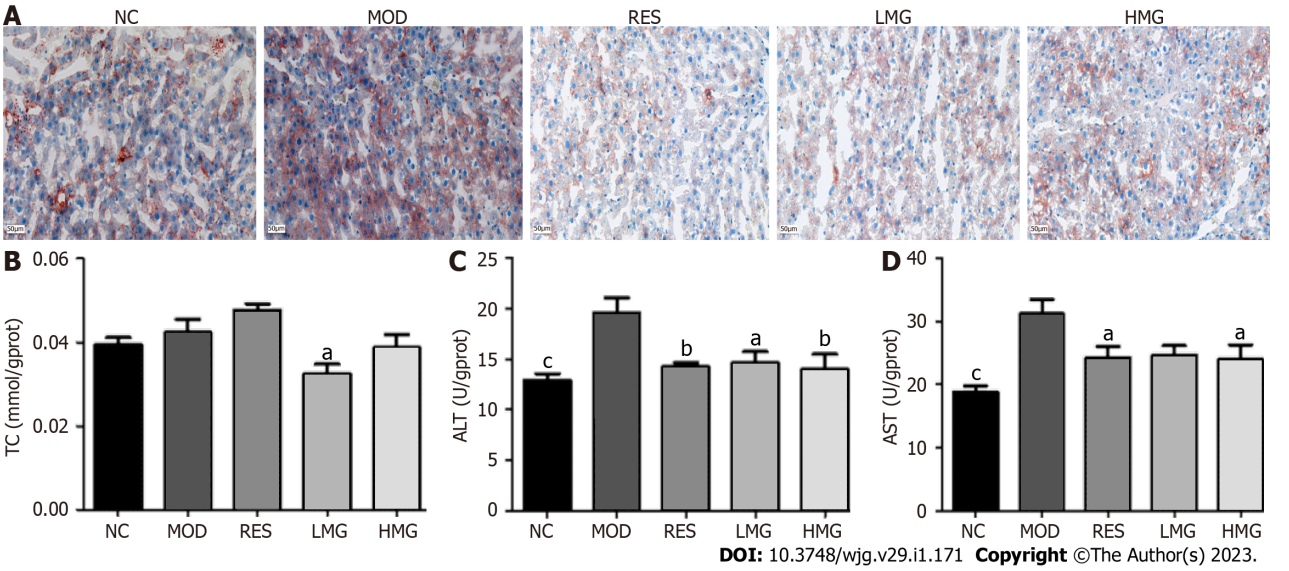
rats were greatly reduced, and the level of MDA increased, which could be reversed by PM treatment. These results suggested that PM extract significantly improved mitochondrial structure and function in the MAFLD process.

In our previous study, the strategy of mitochondrial pharmacology and pharmacochemistry was confirmed to possess the capability to screen the main active constituents of the TCMs regulating the mitochondria to prevent human disease[10]. In the present study, this strategy was used to reveal the pharmacodynamic substances in PM extract remedying mitochondria against MAFLD. Mitochondrial pharmacology confirmed that the PM-derived constituents that regulate oxidative stress and energy production to relieve steatosis of hepatocytes entered the liver mitochondria. Moreover, a total of eight PM-derived monomers that entered the mitochondria of MAFLD rats treated with PM extract were successfully identified by mitochondrial pharmacochemistry, including five prototype components (AE, ED, EG, THSG, and TCS), and three of their metabolites (emodin-glucuronide, emodin-8-O-β-D-glucopyranoside reduction metabolite, and torachrysone-glucuronide). Four monomers have been reported to cure MAFLD, and include AE[22,23], ED[22,24,25], EG[26], and THSG[27]. Our data also show that TCS improved oxidative stress and energy production to prevent steatosis in L02 adipocytes induced by fat emulsion. Furthermore, it was found that the PM-derived monomer group identified from liver mitochondria regulated the state of mitochondria to alleviate MAFLD in HFD-fed mice. Together, these results indicated that the eight substances were potentially major active constituents of PM against MAFLD, thereby validating the capability of mitochondrial pharmacology and pharmacochemistry for efficiently identifying *in vivo* mitochondria-regulated constituents in TCMs.



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Figure 8 Effects of *Polygonum multiflorum*-derived monomer group in liver mitochondria on serum biochemical parameters of high-fat diet-fed mice. Mice were administered saline, *Polygonum multiflorum*-derived monomer groups (30 and 90 mg/kg/d), or resveratrol (18 mg/kg/d). After 3 mo of high-fat diet feeding, mice were sacrificed, and serum was collected. A: Serum levels of total cholesterol were determined using a commercial kit; B: Serum levels of aspartate transaminase were determined using a commercial kit; C: Serum levels of low-density lipoprotein cholesterol were determined using a commercial kit. Values represent the mean ± SD from six animals. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* model group. AST: Aspartate transaminase; HMG: High-dose *Polygonum multiflorum*-derived monomer group; LDL-C: Low-density lipoprotein cholesterol; LMG: Low-dose *Polygonum multiflorum*-derived monomer group; MOD: Model; NC: Normal control; RES: Resveratrol; TC: Total cholesterol.



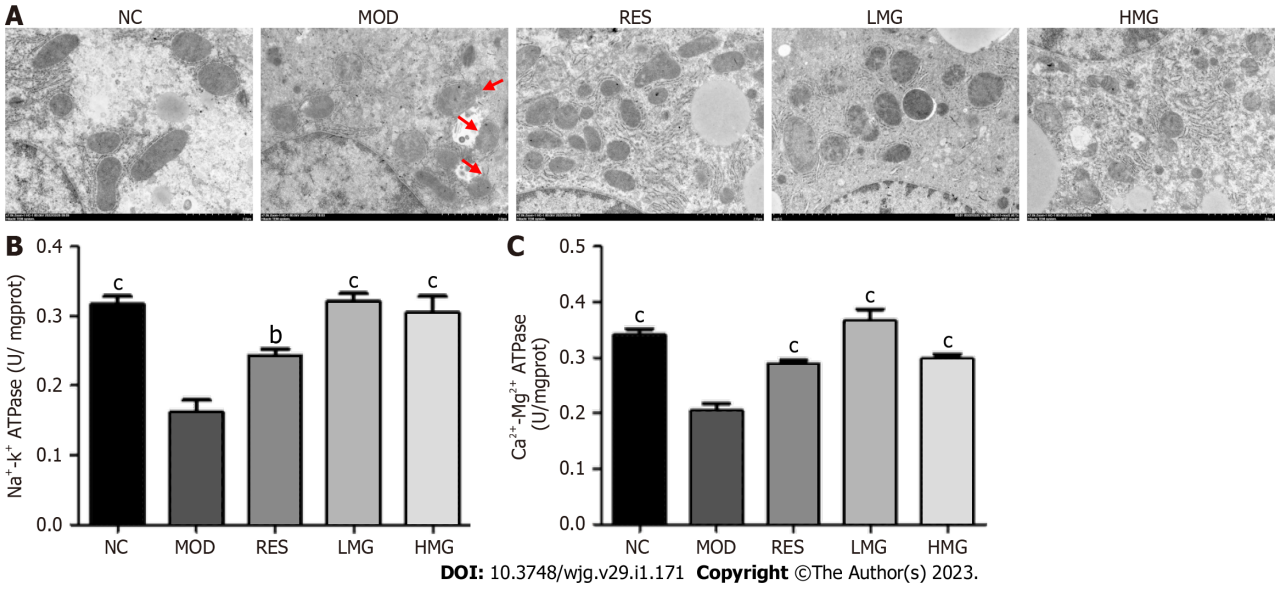
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Figure 9 Effects of *Polygonum multiflorum*-derived monomer group in liver mitochondria on the liver lipid content of high-fat diet-fed mice. Mice were administered saline, *Polygonum multiflorum*-derived monomer groups (30, and 90 mg/kg/d), or resveratrol (18 mg/kg/d). After 3 mo of high-fat diet feeding, mice were sacrificed, and livers were collected. A: Liver histology after Oil Red O staining was observed under a light microscope (× 200); B: Liver levels of total cholesterol were determined using a commercial kit; C: Liver levels of alanine transaminase were determined using a commercial kit; D: Liver levels of aspartate transaminase were determined using a commercial kit. Values represent the mean ± SD from six animals. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* the relevant model group. ALT: Alanine transaminase; AST: Aspartate transaminase; HMG: High-dose *Polygonum multiflorum*-derived monomer group; LMG: Low-dose *Polygonum multiflorum*-derived monomer group; MOD: Model; NC: Normal control; RES: Resveratrol; TC: Total cholesterol.

It is noted that the effects of PM extracts and their components on the detected indices did not show dose-dependence, which is similar to many other TCMs[20]. This may be caused by the complicated substances and action mechanisms of the TCMs, such as multi-components, multiple acting targets, and multiple ways[20]. Additionally, the doses of PM extract and its ingredients used in this study were likely not within the dose-dependent range. In addition, due to the insufficient liver samples, the depth analysis of mechanisms that PM extract and its monomer group regulated mitochondrial status to alleviate MAFLD in HFD-fed animals was not performed, which merits further investigation.

CONCLUSION

The data obtained in this study showed that PM extract restored mitochondrial structure and function and alleviated MAFLD caused by HFD, which may be related to the mitigation of oxidative stress-induced damage and the improvement of energy production. The eight substances obtained by the strategy may be the main bioactive ingredients in PM that regulate mitochondria to relieve MAFLD.



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Figure 10 Effects of *Polygonum multiflorum*-derived monomer group in liver mitochondria on mitochondrial status in the liver from high-fat diet-fed mice. Mice were administered saline, *Polygonum multiflorum*-derived monomer groups (30, and 90 mg/kg/d), or resveratrol (18 mg/kg/d). After 3 mo of high-fat diet feeding, mice were sacrificed, and livers were harvested for mitochondrial ultrastructure observations and mitochondrial isolations. A: Mitochondria were assessed by transmission electron microscope (original magnification × 30000; bar = 500 nm); B: Levels of Na^+-K^+-ATPase in isolated liver mitochondria were determined using a commercial kit; C: Levels of Ca^{2+}-Mg^{2+}-ATPase in isolated liver mitochondria were determined using a commercial kit. Values represent the mean ± SD from six animals. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$ *vs* the relevant model group. ATPase: ATP synthase; HMG: High-dose *Polygonum multiflorum*-derived monomer group; LMG: Low-dose *Polygonum multiflorum*-derived monomer group; MOD: Model; NC: Normal control; RES: Resveratrol.

Thus, PM treatment provided a new method to prevent MAFLD-related mitochondrial dysfunction from alleviating MAFLD. Mitochondrial pharmacology and pharmacochemistry were shown to be efficient strategies for recognizing the bioactive ingredients of TCMs that alleviate mitochondria to prevent disease.

ARTICLE HIGHLIGHTS

Research background

Metabolic dysfunction-associated fatty liver disease (MAFLD) is a serious threat to human health. Mitochondrial dysfunction is a mechanism involved in MAFLD. Modulation of mitochondrial function may become a novel strategy for the treatment of MAFLD. For centuries, *Polygonum multiflorum* (PM) has been used as a common traditional Chinese medicine and nutritional ingredient in China and has been proven to remedy mitochondria and further relieve MAFLD.

Research motivation

To date, the main pharmacodynamic ingredients of PM for regulating mitochondria against MAFLD remain unclear.

Research objectives

To investigate the pharmacodynamic ingredients for the mitochondrial remedy action of PM against high-fat diet (HFD)-induced MAFLD in rats.

Research methods

Fat emulsion-induced L02 adipocyte model and HFD-induced MAFLD rat model were used to evaluate the anti-MAFLD ability of PM and the mechanism of action involved. The adipocyte model was also used to determine the activities of PM-derived constituents in liver mitochondria from HFD-fed rats (mitochondrial pharmacology). PM-derived constituents in liver mitochondria were recognized by ultra-high performance liquid chromatography/mass spectrometry (mitochondrial pharmacochemistry). The abilities of the PM-derived monomer and the monomer group were evaluated by adipocyte model and MAFLD mouse model, respectively.

Research results

PM repaired mitochondrial ultrastructure and prevented oxidative stress and energy production disorder of mitochondria to mitigate fat emulsion-induced cellular steatosis and HFD-induced MAFLD. PM-derived constituents that entered the mitochondria inhibited oxidative stress damage and improved energy production against cellular steatosis. Eight chemicals were found in the mitochondria of PM-administrated rats. The anti-steatosis ability of one monomer and the anti-MAFLD activity of the monomer group were validated.

Research conclusions

PM restored mitochondrial structure and function and alleviated MAFLD, which may be associated with the remedy of oxidative stress and energy production. The identified eight chemicals may be the main bioactive ingredients in PM that adjust mitochondria to prevent MAFLD.

Research perspectives

PM treatment provided a new approach to prevent MAFLD-related mitochondrial dysfunction from alleviating MAFLD. Mitochondrial pharmacology and pharmacochemistry are efficient strategies for identifying the bioactive ingredients of traditional Chinese medicines regulating mitochondria to prevent disease.

FOOTNOTES

Author contributions: Yu LP, Li YJ, and Wang T contributed equally to this work; Li YJ wrote the manuscript; Li YJ, Yu LP, and Wang T performed the experiments; Tao YX and Zhang M provided technical support and suggestions; Yang XX, Yu LP, Zhang M, and Gu W participated in writing and modifying the manuscript; Yang XX and Yu J designed the study; and all authors approved the final manuscript.

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Country/Territory of origin: China

ORCID number: Li-Ping Yu 0000-0003-3914-0003; Yan-Juan Li 0000-0003-3377-8014; Tao Wang 0000-0002-8863-0853; Jie Yu 0000-0001-8100-8896; Xing-Xin Yang 0000-0001-6594-772X.

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ORIGINAL ARTICLE

Observational Study

Impact of *Helicobacter pylori* virulence markers on clinical outcomes in adult populations

Halim Roshrosh, Hanan Rohana, Maya Azrad, Tamar Leshem, Segula Masaphy, Avi Peretz

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Halim Roshrosh, Segula Masaphy, Applied Mycology and Microbiology, Migal, Kiryat Shemona 1101202, Israel

Hanan Rohana, Maya Azrad, Tamar Leshem, Avi Peretz, Department of Microbiology, Padeh Poriya Medical Center, Tiberias 111508, Israel

Corresponding author: Maya Azrad, PhD, Research Scientist, Department of Microbiology, Padeh Poriya Medical Center, Lower Galillee Israel, Tiberias 111508, Israel.
mazrad@poria.health.gov.il

Abstract

BACKGROUND

In recent years, associations between specific virulence markers of *Helicobacter pylori* (*H. pylori*) and gastrointestinal disorders have been suggested.

AIM

To investigate the presence of virulence factors including vacuolating cytotoxin A genotypes (*s1m1*, *s1m2*, *s2m1*, and *s2m2*), cytotoxin-associated gene A (*Cag*A), and urease activity in *H. pylori* strains isolated from Arab and Jewish populations in northern Israel and to assess associations between these factors and patients' demographics and clinical outcomes.

METHODS

Patients (n = 108) who underwent gastroscopy at the Baruch Padeh Medical Center, Poriya due to symptomatic gastroduodenal pathologies as part of *H. pylori* diagnosis were enrolled in the study. Gastric biopsy specimens were collected from the antrum of the stomach. Clinical condition was assessed by clinical pathology tests. Bacteria were isolated on modified BD Helicobacter Agar (BD Diagnostics, Sparks, MD, United States). Bacterial DNA was extracted, and PCR was performed to detect *Cag*A and vacuolating cytotoxin A genes. Urease activity was assessed using a rapid urease test.

RESULTS

A significant correlation was found between disease severity and patient ethnicity (P = 0.002). A significant correlation was found between *Cag*A presence and the *s1m1* genotype (P = 0.02), which is considered the most virulent genotype. Further, a higher level of urease activity was associated with isolates originating from the Jewish population. Moreover, higher urease activity levels were

measured among *Cag*A-/*s1m1* and *Cag*A-/*s2m2* isolates.

CONCLUSION
Our study highlights the importance of incorporating molecular methods for detection of virulence markers of *H. pylori* in order to tailor optimal treatments for each patient. Further investigation should be performed regarding associations between *H. pylori* virulence factors and ethnicity.

Key Words: *Helicobacter pylori*; Virulence factors; Vacuolating cytotoxin A; Cytotoxin-associated gene A; Urease activity

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Core Tip: In recent years, associations have been found between virulence markers of *Helicobacter pylori* and gastrointestinal disorders. In parallel, several physicians in northern Israel noted a higher treatment failure rate among Arab patients compared to Jewish patients. This work found a significant correlation between disease severity and patient ethnicity ($P = 0.002$). Further, a higher level of urease activity was associated with isolates originating from the Jewish population. Moreover, higher urease activity levels were measured among *Cag*A-/*s1m1* and *Cag*A-/*s2m2* isolates. These findings are expected to advance personalization of treatment to specific strains based on their virulence factors.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a microaerophilic, Gram-negative bacterium that colonizes the gastric mucosa and infects the stomach epithelium, causing peptic ulcer disease[1]. Unrelenting *H. pylori* infections cause chronic inflammation, which can result in gastritis, intestinal metaplasia, and even gastric cancer[1].

The genetic variability of *H. pylori* and its host, combined with environmental factors, have been suggested to affect the clinical outcome[1-3]. *H. pylori* pathogenesis is mediated *via* distinct virulence factors, including the secreted vacuolating cytotoxin A (*Vac*A), cytotoxin-associated gene A (*Cag*A) protein, and urease[1,2]. VacA is one of the main toxins secreted by the bacterium; once bound to host cells and internalized, it causes "vacuolation," characterized by the accumulation of large vesicles that disrupt protein trafficking pathways. Two polymorphic regions in the *Vac*A gene sequence are the signal sequence region (s-region) and the mid region (m-region), with allelic variations classified as *s1* or *s2* and *m1* or *m2*, respectively[3]. It was suggested that isolates with a *s1/m1* genotype cause more severe chronic inflammation compared to the other *Vac*A genotypes[4].

H. pylori strains can be divided into two main subpopulations according to their ability to produce CagA, a 120-145 kDa protein associated with gastric carcinoma. The protein interferes with cellular signal transduction, provoking cellular dysfunction that can eventually lead to cell transformation and cancer[5].

Along with VacA and CagA, urease activity in *H. pylori* is an indicator of bacterial virulence. Urease is a metalloenzyme that requires nickel for its activity and consists of two components, UreA and UreB. The enzyme breaks down urea into ammonia and carbon dioxide, promoting bacterial survival in the acidic environment of the stomach. In addition, urease is involved in *H. pylori* colonization in the gastric tissue, prevention of phagocytosis, and induction of proinflammatory cytokines[6,7].

In addition to the virulence factors that may affect the efficacy of *H. pylori* eradication regimens, antibiotic resistance poses a major challenge to treatment success. Several physicians in northern Israel noted that the Arab population has a higher treatment failure rate compared to the Jewish population, a finding which triggered a study that examined antibiotic resistance of *H. pylori* among these two groups in Israel[8]. Indeed, isolates from the Arab population were more resistant to both clarithromycin and levofloxacin and exhibited simultaneous resistance to more antibiotics as compared to isolates from Jewish patients[8]. The current study further examined the characteristics of *H. pylori* in adult Arab and Jewish populations in northern Israel, with an emphasis on the three virulence markers of *H. pylori* and their correlation with clinical outcomes.

MATERIALS AND METHODS

Study population

The study group included 108 isolates of patients who underwent gastroscopy at the Baruch Padeh Medical Center, Poriya, due to symptomatic gastroduodenal pathologies as part of the *H. pylori* workup between November 2018 and December 2019. We included the first 108 biopsies from which *H. pylori* was successfully isolated at the microbiology laboratory of the medical center. The study was approved by the Helsinki Committee of the Baruch Padeh Medical Center, Poriya (Approval no. POR 0007-20). The Institutional Review Board committee waived the need for participant approval. Gastric biopsy specimens were collected from the antrum of the stomach. Clinical pathology tests were performed to assess the patients' clinical conditions. Demographic and clinical data were retrospectively collected from the patients' medical records.

Histology staining and pathology

Gastric biopsy specimens were stained using hematoxylin and eosin staining method in order to identify *H. pylori* and to evaluate the degree of inflammation. The bacterium has an actively dividing spiral shape that changes to coccoid morphology under stressful environments.

All histologic slides were reviewed by a single blinded gastrointestinal pathologist at the pathology laboratory of the Baruch Padeh Medical Center, Poriya and graded unremarkable (none), mild, moderate, or severe, based on the presence of acute (polymorphonuclear cells) or chronic (monocytes, lymphocytes, plasma cells) inflammation, lymphoid aggregates, and metaplasia.

Bacterial isolation and identification

H. pylori identification was carried out in accordance with the routine identification tests of the clinical microbiology laboratory including a Gram stain, oxidase, catalase, and urease tests.

Biopsy specimens were manually minced with a sterile scalpel, seeded on modified BD Helicobacter Agar plates (BD Diagnostics, Sparks, MD), and incubated for 7 d at 35 °C in a microaerobic atmosphere (5% O_2 and 10% CO_2) produced by a gas-generating system adapted for *Campylobacter* (CampyGen™; Gamidor Diagnostics, Petah Tikva, Israel).

Final identification of the bacteria was performed by matrix-assisted laser desorption ionization-time of flight mass spectrometry[9], using the Bruker Biotyper system (Bruker Daltonics, Bremen, Germany) with MALDI BIOTYPER 3.3 (Bruker Daltonics) software.

Molecular characterization of genes associated with bacterial virulence

DNA extraction: Tissue collected from gastroscopic biopsy was finely chopped with a sterile scalpel and then lysed by tissue lysis buffer supplemented with proteinase K enzyme (Bioneer, Daejeon, Korea). Total DNA was extracted using the AccuPrep Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea), according to the manufacturer's instructions.

Multiplex PCR assay: DNA was amplified with a multiplex PCR, designed to detect the *Vac*A and *Cag*A genes in a single run, using specific primers (Table 1). For this purpose, 10 μL Taq ReadyMix2 (Hy labs, Rehovot, Israel) was added to 0.2 μL forward primer, 0.2 μL reverse primer, 4 μL template DNA, and 5.6 μL nuclease-free water. Reaction conditions were 35 cycles of: Denaturation of the pre-amplified templates at 95 °C for 1 min, followed by an annealing step at 72 °C for 1 min, an extension step at 25 °C for 1 min and one additional extension step for 7 min. PCR products were visualized by 1% agarose gel electrophoresis.

Urease activity

Urease activity was quantified using the rapid urease test in a 96-well plate. For this purpose, bacteria were placed in a sterile Eppendorf tube containing sterile physiological solution until 0.5 McFarland turbidity was reached. Rapid Urease Test Brute solution (100 μL; Novamed, Jerusalem, Israel), containing 2% urea, pH 6.8, was then added to each well, along with 100 μL of the bacterial stock. Then, absorbance at 570 nm (optical density, $O.D_{570}$) was measured after 1 min, 5 min and 10 min using the Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, Waltham, MA, United States)[10]. A change in the solution color from orange to pink was considered a positive result.

Statistical analysis

Continuous variables (age, urease activity) were presented as means and ranges with standard deviations and categorical variables (sex, disease severity, ethnicity, living area, genotypes) were presented as absolute numbers and percentages. *T* test or one-way analysis of variance was used to compare continuous variables of two or more groups and Fisher's exact test to assess the relationship between categorical variables. *P* value < 0.05 indicated statistical significance. Statistical analysis was performed using R Statistical Software (version 4.1; R Foundation for Statistical Computing, Vienna, Austria).

Table 1 List of genes and primer sequences used in the study

Primer	Gene	Primer sequence	Product size (bp)
CAGAF	*Cag*A	5′-GATAACAGGCAAGCTTTTGAGG-3′	349
CAGAR		5′-CTGCAAAAGATTGTTTGGCAGA-3′	
VA1-F	*Vac*A signal region	5′-ATGGAAATACAACAAACACAC-3′	259/286 (s1/s2)
VA1-R		5′-CTGCTTGAATGCGCCAAAC-3′	
VAG-F	*Vac*A middle region	5′-CAATCTGTCCAATCAAGCGAG-3′	567/642 (m1/m2)
VAG-R		5′-GCGTCTAAATAATTCCAAGG-3′	

*Vac*A: Vacuolating cytotoxin A; *Cag*A: Cytotoxin-associated gene A.

RESULTS

Demographic characteristics

In total, 108 patients [average of 42.3 (18.0-88.0) years] were enrolled in this study, 24% of whom were males and 76% of whom were females (Table 2). Within the study group, 56 were Arabs and 52 were Jews; 61.1% lived in a city and 33.9% lived in a village. A larger percentage of the Arab *vs* Jewish cohort lived in villages (62.5% *vs* 13.5%, respectively).

VacA and CagA genotypes

To characterize the common *Cag*A and *Vac*A genotypes in the patient population, 108 isolates were randomly selected, of which 56 originated from the Arab cohort and 52 from the Jewish cohort (Table 3). *Cag*A was identified in 24 (22.2%) samples and was distributed equally between the two ethnic groups. However, it had a higher frequency among isolates from patients living in villages (67.7%) compared to isolates from those living in cities (33.3%) (P = 0.149). The most prevalent *Vac*A genotype was *s2m1*. No statistically significant associations were noted between the different genotypes and demographic characteristics.

A significant association was found between the presence of *Cag*A and specific *Vac*A genotypes (P = 0.002) (Figure 1); 33.3% of the *Cag*A-positive strains had the *s1m1* genotype, which is considered the most virulent genotype[7]. Additionally, 61.9% of the *Cag*A-negative strains had the *s2m1* genotype.

Urease activity

Urease activity was found to be faster in isolates from the Jewish population compared to isolates from the Arab population. This was observed at all tested time points (1 min, 5 min, and 10 min; $P < 0.05$ for all). The results of the first-minute measurements are presented in Figure 2. No significant associations were found between urease activity and patient's sex, place of residence and the presence of the *Cag*A gene.

In addition, urease activity in *Cag*A (-) *s2m2* strains (mean O.D 0.33) and in *Cag*A (-) *s1m1* isolates (mean O.D 0.32) were significantly higher than urease activity in *Cag*A (-) *s1m2* (mean O.D 0.28), (P = 0.013 and P = 0.016, respectively) (Figure 3).

Associations between virulence factors, patient ethnicity and disease severity

A significant association was found between disease severity and ethnicity (P =0.002) (Table 4); in patients with mild disease, 58% were Jews while 42% were Arabs. In patients with moderate disease, 75.7% were Arabs compared to 24.3% Jews. Finally, the severe group included 66.7% Arabs compared to 33.3% Jews. No significant links were found between disease severity and urease activity, *Cag*A gene occurrence, *Vac*A alleles, and genotype combinations.

DISCUSSION

The main purpose of this study was to evaluate and compare the virulence markers of *H. pylori* among two adult populations in northern Israel and their correlation with clinical outcomes. It should be noted that, due to its special growth requirements and slow growth, cultivation of *H. pylori* is difficult. As a result, the diagnosis of *H. pylori* infection is usually performed, as opposed to diagnosis of other bacterial infections, on the basis of indirect tests that do not require bacterial isolation[11]. This is the reason why there is limited data on the distribution of virulence factors and their associations with clinical outcomes among *H. pylori* isolates in Israel.

Table 2 Demographic characteristics of the study participants, *n* (%)

Characteristic	Arabs, *n* = 56	Jews, *n* = 52	Total, *n* = 108
Sex			
Male	14 (25)	39 (23)	26 (24)
Female	84 (75)	64 (77)	82 (76)
Age, yr			
Average (range)	40.93 (18-81)	43.65 (18-88)	42.30 (18-88)
Area of residence			
City	21 (37.5)	45 (86.5)	66 (61.1)
Village	35 (62.5)	7 (13.5)	42 (33.9)

Table 3 The prevalence of vacuolating cytotoxin A alleles and cytotoxin-associated gene A *vs* patient demographics

Characteristic	*CagA*-positive, *n* = 24	*CagA*-negative, *n* = 84	*P*	*VacA* genotype				*P*
				s1m1	*s1m2*	*s2m1*	*s2m2*	
Ethnicity			0.834					0.729
Arabs	12 (50.0)	40 (47.6)		11 (21.2)	11 (21.2)	27 (51.9)	3 (5.8)	
Jewish	12 (50.0)	44 (52.4)		8 (14.3)	15 (26.8)	31 (55.4)	2 (3.6)	
Sex			0.623					0.214
Male	7 (29.2)	29 (34.5)		6 (16.7)	5 (13.9)	22 (61.1)	3 (8.3)	
Female	17 (70.8)	55 (65.5)		13 (18.1)	21 (29.2)	36 (50.0)	2 (2.8)	
Residence			0.149					0.055
Village	16 (67.7)	42 (50.0)		8 (13.8)	17 (29.3)	28 (48.3)	5 (8.6)	
City	8 (33.3)	42 (50.0)		11 (22.0)	9 (18.0)	30 (60.0)	0 (0)	

*Vac*A: Vacuolating cytotoxin A; *Cag*A: Cytotoxin-associated gene A.

*Cag*A was found in 22.2% of the detected strains. This rate is a bit lower than expected according to previous studies; the prevalence of the cytotoxin-associated gene pathogenicity island, which includes *Cag*A, varies between different geographic areas, ranging from 95% in Western and South Africa and East and Central Asia to 28% in Latin America. In Europe, approximately 58% of the *H. pylori* strains carry the *Cag*A gene, while in the Middle East, *CagA* was detected in approximately 50% of the strains [12,13]. As only a sample of our isolates were tested for the presence of *Cag*A, it is possible that a higher prevalence of this gene exists among *H. pylori* strains in Israel.

We did not find significant differences in the distribution of *Cag*A between the two populations. Interestingly, Muhsen *et al*[14], who also investigated *H. pylori* isolates in Arab and Jewish populations, demonstrated higher *Cag*A IgG antibodies in the Arab population. Another study, performed in Israeli children and adolescents, found higher *H. pylori* seroprevalence among Arab participants as compared to Jewish participants[15].

Regarding the virulence factor *Vac*A, *s2m1* genotype was the most common, present in 53.3% of the isolates, while only 17.6% carried the *s1m1* genotype. In a study conducted in South Africa, *s1m1* was the most common genotype (56.4%), while the *s2m1* genotype was present in only 10.3%[16]. In a similar study conducted in Iran, the most common genotype was *s2m2*, found in 50% of the isolates[17]. It should be noted that the frequency of the alleles in different populations is influenced by several factors, including evolution, natural selection, mutations, and genetic drift[18].

*Cag*A was identified most frequently together with the *Vac*A genotype *s1m1* (P = 0.02), which is considered the most virulent[4]. This result reinforces evidence from a previous study, which found that most *Vac*A s1 strains carry *Cag*A as well[19,20]. Previous studies suggested that the presence of *Cag*A together with certain genotypes of *Vac*A can indicate disease severity. For example, its appearance with the genotype *s1m2* or *s1m1* was correlated with the appearance of peptic ulcers, while its appearance with *s2m2* was correlated with gastritis[17]. In light of the above, it is important to profile the *Vac*A and *Cag*A variants in patients in order to assess disease progression. This may aid in optimizing medical treatment.

Table 4 Associations between virulence factors, patient ethnicity, and disease severity, *n* (%)

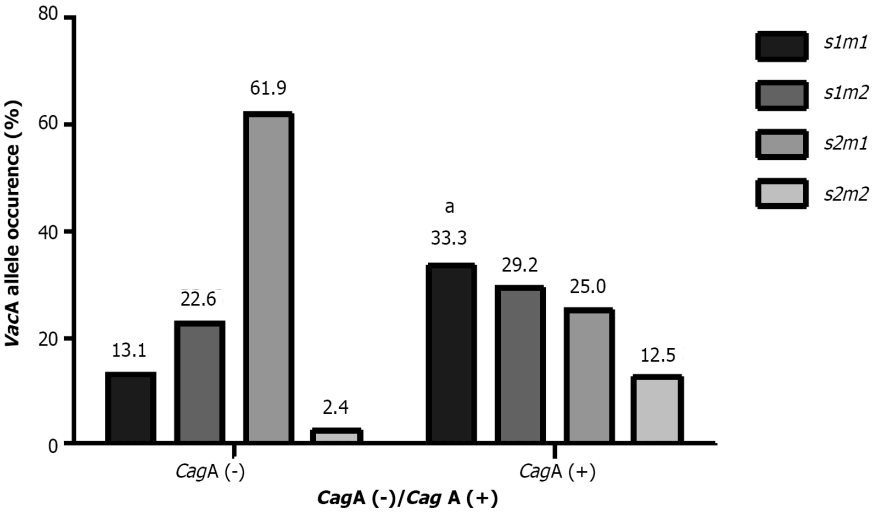
	Disease severity					*P* value
	Unremarkable, *n* = 18	Mild, *n* = 50	Moderate, *n* = 37	Severe, *n* = 3	Total, *n* = 108	
Ethnicity						0.002
Arab	5 (27.8)	21 (42.0)	28 (75.7)	2 (66.7)	56 (51.9)	
Jewish	13 (72.2)	29 (58.0)	9 (24.3)	1 (33.3)	52 (48.1)	
Urease activity						
t1	0.32 (0.05)	0.29 (0.06)	0.29 (0.07)	0.30 (0.06)	0.30 (0.06)	0.273
t5	0.32 (0.05)	0.30 (0.05)	0.29 (0.07)	0.34 (0.02)	0.30 (0.06)	0.174
t10	0.32 (0.05)	0.30 (0.06)	0.30 (0.06)	0.35 (0.04)	0.30 (0.06)	0.171
t15	0.33 (0.04)	0.31 (0.05)	0.31 (0.06)	0.36 (0.03)	0.31 (0.05)	0.245
*Cag*A gene						0.534
*Cag*A-	13 (72.2)	41 (82.0)	27 (73.0)	3 (100)	84 (77.8)	
*Cag*A+	5 (27.8)	9 (18.0)	10 (27.0)	0 (0)	24 (22.2)	
*Vac*A *s* allele						0.651
*Vac*A *s1*	5 (27.8)	22 (44.0)	16 (43.2)	1 (33.3)	44 (40.7)	
*Vac*A *s2*	13 (72.2)	28 (56.0)	21 (56.8)	2 (66.7)	64 (59.3)	
VacA *m* Allele						0.652
*Vac*A *m1*	3 (16.7)	14 (28.0)	7 (18.9)	1 (33.3)	25 (23.1)	
*Vac*A *m2*	15 (83.3)	36 (72.0)	30 (81.1)	2 (66.7)	83 (76.9)	
Genotype						0.926
*Cag*A+/*s1m1*	0 (0)	3 (6.0)	4 (10.8)	0 (0)	7 (6.5)	
*Cag*A+/*s1m2*	1 (5.6)	2 (4.0)	3 (8.1)	0 (0)	6 (5.6)	
*Cag*A+/*s2m2*	2 (11.1)	2 (4.0)	2 (5.4)	0 (0)	6 (5.6)	
*Cag*A+/*s2m1*	1 (5.6)	1 (2.0)	1 (2.7)	0 (0)	3 (2.8)	
*Cag*A-/*s1m1*	2 (11.1)	7 (14.0)	2 (5.4)	1 (33.3)	12 (11.1)	
*Cag*A-/*s1m2*	2 (11.1)	11 (22.0)	7 (18.9)	0 (0)	20 (18.5)	
*Cag*A-/*s2m2*	10 (55.6)	22 (44.0)	18 (48.6)	2 (66.7)	52 (48.1)	
*Cag*A-/*s2m1*	0 (0)	2 (4.0)	0 (0)	0 (0)	2 (1.9)	

*Vac*A: Vacuolating cytotoxin A; *Cag*A: Cytotoxin-associated gene A.

Apart from *Vac*A and *Cag*A, urease activity is another indicator of *H. pylori* virulence. We found a higher urease activity among isolates from the Jewish population as compared to those from Arabs ($P < 0.005$). No previous study has investigated this issue. As urease activity is influenced by specific food ingredients such as isothiocyanates[10] and essential oils[21], variations in nutrition habits may explain the difference in urease activity between isolates from Arab and Jewish patients. Further studies are needed to confirm our result and investigate its meaning.

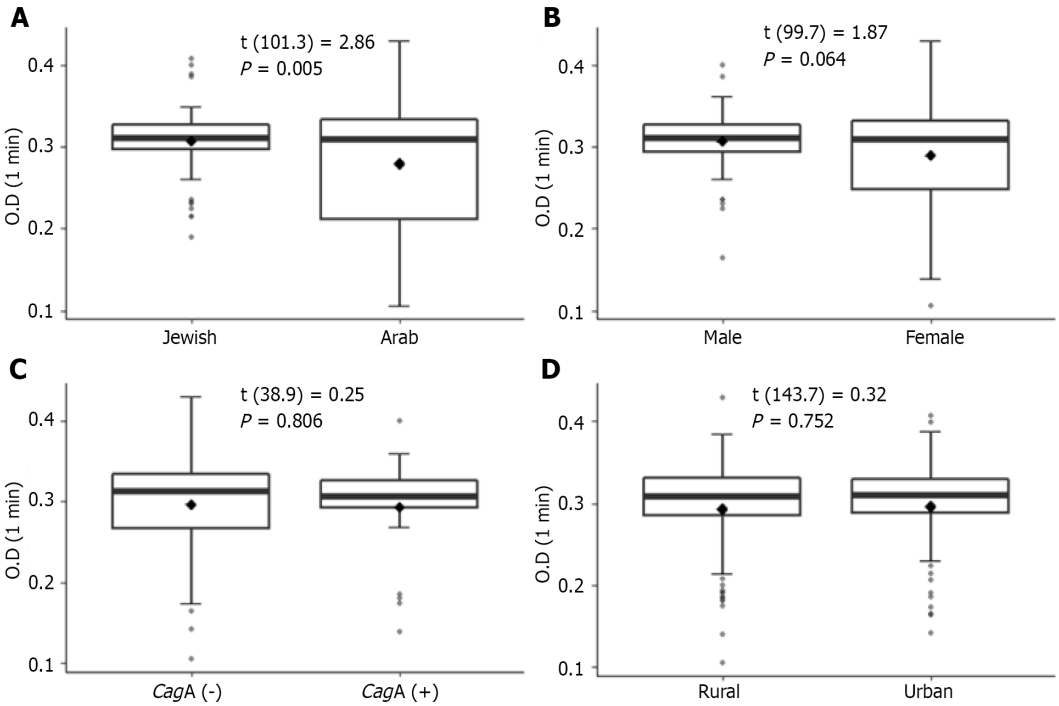
Significant differences in urease activity were noted among isolates with specific *Cag*A and *VacA* genotype combinations. Both *Cag*A (-) *s1m1* and *Cag*A (-) *s2m2* showed the highest urease activity. This result shows that the absence of *Cag*A may result in increased urease activity, especially when combined with the most virulent alleles of the *Vac*A gene, *s1m1*, as previously suggested[6].

Given that antibiotic resistance was found to be higher in the Arab population[8], we cautiously suggest that increased urease activity does not coincide with increased antibiotic resistance. In our preliminary analysis, no significant correlation was found between disease severity and urease activity. These results were quite surprising as previous studies did report on such associations[22]. It is possible that this contradiction is due to our relatively small sample size. However, we did find a significant correlation between ethnicity and disease severity ($P = 0.002$). This finding may be ascribable to differences in health-related lifestyle between different ethnic groups as well as differences in socioeconomic conditions along with cultural and social customs between groups related to their



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Figure 1 Distribution of the different vacuolating cytotoxin A genotypes (percentage) among 108 isolates, according to cytotoxin-associated gene A presence. *Vac*A: Vacuolating cytotoxin A; *Cag*A: Cytotoxin-associated gene A. [a]*P* < 0.05.



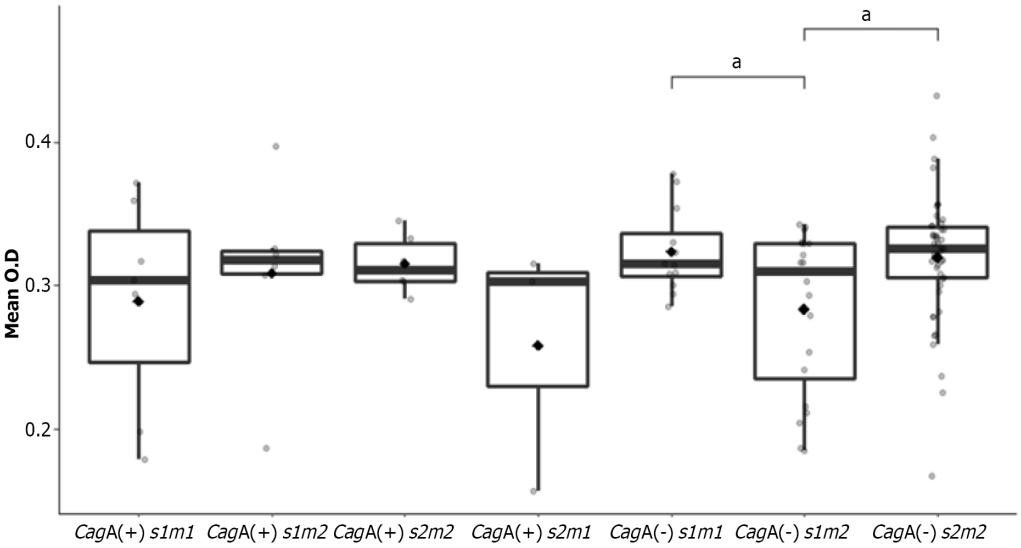
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Figure 2 Urease activity in relation to ethnicity, sex, cytotoxin-associated gene occurrence, and place of residence. A: Ethnicity; B: Sex; C: Cytotoxin-associated A gene occurrence; D: Place of residence. Urease activity (indicated by O.D) was measured 1 min after incubation of bacteria with urea solution, as described in the Materials and Methods section. *Vac*A: Vacuolating cytotoxin A; *Cag*A: Cytotoxin-associated gene A.

residential environments, which can undoubtedly affect morbidity. Furthermore, it can also be explained by the high antibiotic resistance found among the Arab population[8].

CONCLUSION

Our study highlighted the importance of incorporating molecular methods for detection of virulence markers of *H. pylori* in order to tailor optimal treatments for each patient. Further investigation should be performed regarding associations between *H. pylori* virulence factors and ethnicity.



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Figure 3 Urease activity as a function of isolate genotype combination. Urease activity was measured 1 min, 5 min, and 10 min after incubation of bacteria with urea solution, as described in the Materials and Methods section. The minimal, maximal, and median of mean urease activity values are shown (the median is indicated by the line within the bar) per each group of isolates with a different genotype of cytotoxin-associated gene A and vacuolating cytotoxin A genes. O.D: Optical density; *Vac*A: Vacuolating cytotoxin A; *Cag*A: Cytotoxin-associated gene A. $^{a}P < 0.05$.

ARTICLE HIGHLIGHTS

Research background

Helicobacter pylori (*H. pylori*) is a microaerophilic, Gram-negative bacterium that colonizes the gastric mucosa and infects the stomach epithelium, causing peptic ulcer disease. The genetic variability of *H. pylori* and its host, combined with environmental factors, have been suggested to affect the clinical outcome. *H. pylori* pathogenesis is mediated *via* distinct virulence factors, including the secreted vacuolating cytotoxin A, cytotoxin-associated gene A, and urease.

Research motivation

In recent years, associations between specific virulence markers of *H. pylori* and gastrointestinal disorders have been suggested.

Research objectives

To investigate the distribution of three virulence factors among isolates from both Arab and Jewish populations and to assess their impact on clinical presentations.

Research methods

We enrolled 108 patients tested for the presence of vacuolating cytotoxin A and cytotoxin-associated gene A genes and evaluated the urease activity levels. We assessed the clinical state of the patients by hematoxylin and eosin staining of the gastric biopsies from which the bacteria were recovered.

Research results

We found associations between disease severity and ethnicity and between some of the virulence factors to ethnicity.

Research conclusions

Our study highlighted the importance of incorporating molecular methods for detection of virulence markers of *H. pylori* in order to tailor optimal treatments for each patient.

Research perspectives

Further investigation should be performed regarding associations between *H. pylori* virulence factors and ethnicity.

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FOOTNOTES

Author contributions: Roshrosh H, Rohana H, and Peretz A contributed to conceptualization; Roshrosh H, Rohana H, Azrad M, and Leshem T contributed to data curation; Roshrosh H, Rohana H, Azrad M, Leshem T, and Peretz A contributed to formal analysis; Roshrosh H, Rohana H, and Leshem T contributed to investigation and methodology; Leshem T, Azrad M, and Peretz A contributed to project administration and supervision; Roshrosh H, Rohana H, and Leshem T contributed to validation; Roshrosh H, Rohana H, and Leshem T contributed to visualization; Roshrosh H, Rohana H, Azrad M, and Peretz A contributed to writing the original draft; Roshrosh H, Rohana H, Azrad M, Leshem T, and Peretz A contributed to reviewing and editing; All authors read and agreed to the published version of the manuscript.

Institutional review board statement: The study was reviewed and approved by the Helsinki Committee of the Baruch Padeh Medical Center, Poriya (Approval No. POR 0007-20).

Informed consent statement: The Institutional Review Board committee waived the need for participant approval.

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

Data sharing statement: The data presented in this study are available on request from the corresponding author.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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Country/Territory of origin: Israel

ORCID number: Maya Azrad 0000-0002-0966-2575; Avi Peretz 0000-0002-9012-6015.

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SYSTEMATIC REVIEWS

Liver pathology in COVID-19 related death and leading role of autopsy in the pandemic

Martina Zanon, Margherita Neri, Stefano Pizzolitto, Davide Radaelli, Monica Concato, Michela Peruch, Stefano D'Errico

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Martina Zanon, Davide Radaelli, Monica Concato, Michela Peruch, Stefano D'Errico, Department of Medical Surgical and Health Sciences, University of Trieste, Trieste 34149, Italy

Margherita Neri, Department of Medical Sciences, University of Ferrara, Ferrara 44121, Italy

Stefano Pizzolitto, Department of Pathology, Santa Maria della Misericordia University Hospital, Udine 33100, Italy

Corresponding author: Stefano D'Errico, PhD, Assistant Professor, Department of Medical Surgical and Health Sciences, University of Trieste, Strada di Fiume, Trieste 34149, Italy. sderrico@units.it

Abstract

BACKGROUND

Information on liver involvement in patients with coronavirus disease 2019 is currently fragmented.

AIM

To highlight the pathological changes found during the autopsy of severe acute respiratory syndrome coronavirus 2 positive patients.

METHODS

A systematic literature search on PubMed was carried out until June 21, 2022.

RESULTS

A literature review reveals that pre-existing liver disease and elevation of liver enzyme in these patients are not common; liver enzyme elevations tend to be seen in those in critical conditions. Despite the poor expression of viral receptors in the liver, it seems that the virus is able to infect this organ and therefore cause liver damage. Unfortunately, to date, the search for the virus inside the liver is not frequent (16% of the cases) and only a small number show the presence of the virus. In most of the autopsy cases, macroscopic assessment is lacking, while microscopic evaluation of livers has revealed the frequent presence of congestion (42.7%) and steatosis (41.6%). Less frequent is the finding of hepatic inflammation or necrosis (19%) and portal inflammation (18%). The presence of microthrombi, frequently found in the lungs, is infrequent in the liver, with only 12% of cases presenting thrombotic formations within the vascular tree.

CONCLUSION
To date, the greatest problem in interpreting these modifications remains the association of the damage with the direct action of the virus, rather than with the inflammation or alterations induced by hypoxia and hypovolemia in patients undergoing oxygen therapy and decompensated patients.

Key Words: Liver; COVID-19; Autopsy; Immunohistochemistry; *In situ* hybridization; Immunofluorescence

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Core Tip: A literature review, about liver pathology in coronavirus disease 2019 (COVID-19) patients, demonstrates the presence of liver damage, which is represented mainly by congestion, steatosis, hepatic inflammation and necrosis, and portal inflammation. The problem to date is whether the damage is COVID-19 related (meaning from direct virus damage/inflammatory related/systemic pathology related) or drug induced. However, this demonstration involves the need to be careful during drug treatment in patients with altered liver enzyme values to prevent further clinical worsening.

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INTRODUCTION

The new coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has been well studied in relation to pulmonary and cardiac histologic manifestations, but little is yet known regarding hepatic manifestations. COVID-19 has, in fact, to be considered a systemic infectious and inflammatory disease with histological changes also in other organs apart from its main target represented by the lungs. Liver involvement to date is recognized and defined as any liver damage occurring during the course of the disease or its treatment[1], meaning that liver damage can be caused by direct cytotoxicity or inflammatory response and hypoxic/cardio-vascular changes, or it may be drug-induced[2-4]. SARS-CoV-2 liver tropism is also well studied, with many authors demonstrating the presence of angiotensin converting enzyme 2 (ACE2) receptor and transmembrane serine protease 2 in the liver, mainly expressed on cholangiocytes, where the levels of expression are similar to those on alveolar cells, though they are only minimally expressed on hepatocytes. No ACE2 expression was demonstrated on sinusoidal endothelial cells or Kupffer cells, apart from Wanner *et al*[5] who demonstrated minimal expression of ACE2 on Kupffer cells through immunofluorescence and Pirisi *et al*[6] who demonstrated the presence of virus-like particles in endothelial cells of hepatic sinusoids. Curiously, in patients with liver fibrosis/cirrhosis and in cases of hypoxia, the expression of ACE2 is increased, therefore pre-existing liver injury or hypoxic conditions, common in patients with COVID-19, could favor SARS-CoV-2 liver tropism[4,7-9]. Liver infection could also be explained by its immunological role and the proximity to the digestive organs, which exhibit a strong SARS-CoV-2-tropism, that could favor the entry of the virus through the portal system. Hepatic macrophages (mainly Kupffer cells) and sinusoidal endothelial cells have a key role in the activation of the immune response through pathogen recognition receptors, thus favoring virus entry[10].

The incidence of liver injury in COVID-19 patients is seen in 14%-53% cases[9,11,12] mainly demonstrated through abnormal liver function enzymes. In the literature, only a small number of studies focus on liver damage and even fewer on histological changes in patients who died with or from COVID-19. The purpose of this review is to summarize the results of studies in the literature and evaluate the biochemical and histological changes in the liver, demonstrating that the execution of autopsies is not obsolete, but represents a fundamental tool to create a bridge between clinical manifest-ations and cytological damage.

MATERIALS AND METHODS

A systematic literature search on PubMed was carried out until June 21, 2022. No time restrictions were applied. The review was conducted using MeSH terms, Boolean operators, and free-text terms to broaden the research. Studies focusing on autopsies of COVID-19 deaths and in particular on liver

pathology were initially searched using the terms "((COVID-19) AND (autopsy) AND ((death) OR (liver))" in title, abstract, and keywords. Study design included case reports, case series, and retrospective and prospective studies. Reviews were excluded in order not to create duplication of data, but were analyzed to search for any studies not resulting from the search in the database. No unpublished or gray literature was searched. A total of 526 articles were found in the database. The evaluation of references during full text screening allowed the inclusion of further seven studies. After evaluation of abstracts and full text, 46 articles were included because of their compliance with the inclusion criteria. We also conducted a relevant search using Reference Citation Analysis (https://www.referencecitationanalysis.com/) database to supplement and improve the highlights of the latest cutting-edge research results. Data from each included study were extracted using Microsoft Excel spreadsheets, including information on authors, publishing year, nation, sample size, gender, age, type of autopsy, laboratory results, pre-existing liver disease, macroscopic and microscopic results, additional staining, cause of death, medications, and search of the virus in the liver (Table 1).

RESULTS

Demographics

A total of 11 case reports and 35 case series were analyzed, with a total of 994 autopsy cases of COVID-19 patients. Studies were from all over the world: One from Hungary, Romania, Japan, South Africa, and United States in association with Brazil each, two from Austria, Belgium, India, Iran, and Turkey each, three from the United Kingdom, four from Italy, five from Germany, Switzerland, and China each, and nine from the United States. Gender was specified in 882 cases, of whom 54% (540) were male and 35% (342) were female. Age ranged from 18 to 102 years with a mean age of 53 years. Age distribution is summarized in Figure 1.

Liver disease

Pre-existing liver diseases were described in 61 (6%) cases, comprising 28 cases of fatty liver disease, 19 cases of chronic liver disease, 11 cases of cirrhosis, and 1 case each of hepatitis B and C. In 161 cases, body mass index (BMI) was over 30 kg/m^2.

Laboratory findings

Laboratory values of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were described in 350 cases, with only 1 case described with values within the ranges of normality. The description of the laboratory values differed somewhat between the various studies, with only 5 studies (55 cases) reporting AST and ALT values at admission and 8 (64 cases) reporting the maximum values during hospitalization. Additional 4 reports for AST (51 cases) and 5 papers for ALT (61 cases) described the laboratory values without specifying the timing of the sampling. Data is summarized in Table 2. Abnormal AST and ALT values were described in 105 and 91 additional cases, respectively.

Hospitalization and medications

For the subsequent analysis of the macroscopic and microscopic findings, it was decided to evaluate whether the patients were hospitalized and whether drug therapies capable of causing liver alterations, such as antibiotics, antivirals, and quinine, were administered. In 861 cases the place where the death took place was described. In 752 cases the patient was hospitalized and died in hospital, 76 cases died at home, 22 cases died in community settings, and 11 cases were not hospitalized and died in other circumstances such as car accidents and falls from a height. In 133 cases a hospital stay or the place of death was not described. Medication administration was described in 201 cases, of which 22 were administered with hydroxychloroquine only. In 41 cases quinine was administered together with an antibiotic or antiviral, in 17 cases antibiotics and antivirals were given, and in 56 only an antibiotic was administered. In 766 cases the administration of hepatotoxic drugs was not reported.

Type of autopsy

Autopsies were performed in all 994 cases; in 508 (51%) cases autopsies were complete, of which 2% (22) had a complete autopsy without the evaluation of the brain to avoid the risk of COVID-19 infection, in 38% (372) of the cases a core biopsy was performed, in 51 (5%) cases a partial autopsy was carried out, and in 41 cases information about the type of autopsy performed was not reported.

Macroscopic results

Macroscopic results were described in only 265 (27%) cases. The most frequent finding, in 79 cases, was the presence of congestion, followed by steatosis in 39 cases. A nutmeg or yellow aspect of the liver surface was seen in 16 cases, a fibrosis-indurated consistency in 6 cases, and only 1 case showed the macroscopic presence of cancer. Lastly, 11 livers were described of increased size (hepatomegaly) and 10 livers as normal. For 144 patients weight was reported; mean weight was 1805 g with a range from 520

Table 1 Literature review results

Ref.	Country	Cases (*n*)	Age (mean, range)	Sex	Type of autopsy	Pre-existing liver disease or other diseases	Laboratory findings	Macroscopi-c results	Microscopi-c results	Additional stainings	Cause of death	Medications	Hospitalizat-ion	Virus identificatio-n in liver
Aguiar *et al* [13], 2020	Switzerland	1	31	F	Complete	Obesity	NR	Nutmeg appearance	Microabs-cesses	None	Respiratory failure in COVID-19	None	Home death	No search
Arslan *et al* [14], 2021	Turkey	7	56, 43-68	M 6, F 1	Partial	3 obesity, 2 hypertension, 1 in hemodialysis	NR	NR	4 mild steatosis, 1 biliary microhamart-oma	None	Respiratory failure in COVID-19	NR	5 hospit-alized, 2 NR	No search
Barton *et al* [15], 2020	United States	2	59, 42-77	M 2	Complete	2 obesity, 1 hypertension and 1 myotonic muscular dystrophy	NR	Case 1: weight: 2232 g, steatosis. Case 2: weight: 1683 g, cirrhosis	Nr	None	1 respiratory failure in COVID-19, 1 complic-ations of hepatic cirrhosis	NR	1 hospit-alized and 1 home death	No search
Beigmo-hammadi *et al*[16], 2021	Iran	7	68, 46-84	M 5, F 2	Core-biopsy	4 hypertension, 1 immuno-compromised and 1 valvular hearth disease	NR	NR	7 congestion, 7 steatosis, 7 portal inflam-mation, 7 hepatitis, 4 ballooning degeneration of hepatocytes, 2 bile plugs, 7 focal confluent necrosis, 4 focal hepatocyte drop out	Masson's trichrome: 1 case of mild fibrosis	NR	7 were treated with hydroxychol-orquine and 6 with antivirals	All hospit-alized	No search
Bradley *et al* [17], 2020	United States	14	74, 42-84	M 6, F 8	7 partial and 7 complete	5 obesity, 8 hypertension, 4 heart failure, 8 CKD	NR	Congestion	10 congestion, 9 steatosis, 1 toxic or metabolic disease, 4 centrilobular necrosis, 3 periportal inflammation	None	12 respiratory failure in COVID-19, 2 cardiovascula-r failure	NR	All hospit-alized	2 positive and 1 negative PCR-test, 11 not tested. 14 negative IHC and TEM

Bryce *et al* [18], 2020	United States	92	NR	NR	Complete	28 fatty liver disease	NR	NR	8 cirrhosis, 57 early organizing thrombi in portal venules and terminal hepatic venules, 41 congestion with some cases showing hemophago-cytosis	None	NR	NR	NR	No search
Bösmüller *et al*[19], 2020	Germany	4	72, 59-79	M 3, F 1	Complete-no brain	2 HIV, 2 hypertension	3 NR 1 with normal values	1 hepato-spleno-megaly and 1 yellowish surface	Case 1: congestion and activation of Kupffer cells. Case 2: macrophages activation with signs hemophago-cytosis	None	1 respiratory failure in COVID-19, 3 MOF	1 had a MOF and was intubated, 1 was treated with meropenem, 1 was treated with ECMO	3 hospit-alized and 1 home death	Negative PCR-test
Bugra *et al* [20], 2021	Turkey	100	55, 7-98	M 80, F 20	Partial	NR	NR	NR	84 inflam-mation, 54 steatosis, 19 glycogen-isation, 9 centilobulary necrosis, 18 autolysis, 45 congestion, 7 endotheliitis, 1 cirrhosis, 2 fibrin thrombosis, 2 bridging necrosis, 1 granulo-matous inflam-mation, 23 cholestasis	CD3+ in portal space	74 respiratory failure in COVID-19 and 26 NR	NR	25 hospit-alized, 55 home dead, 6 falling from height, 5 car accidents and 9 NR	No search
Chornenkyy *et al*[21], 2021	United States	8	58, 18-81	M 3, F 5	Complete	2 chronic liver disease (1 HCV and 1 autoimmune hepatitis), 6 obesity, 4 hypertension	Peak AST: 146 (20-1470) and ALT: 214 (10-9961)	Yellowish surface and congestion	3 periportal fibrosis, 2 necrosis, 5 inflam-mation, 7 portal inflam-	NR	NR	NR	All hospit-alized	4 positive and 4 negative PCR-test

								mation, 6 congestion, 4 steatosis, 6 acute hepatitis					
Danics *et al* [22], 2021	Hungary	100	75, 40-102	M 50, F 50	Complete	36 obesity, 6 liver diseases, 85 hypertension	41 elevated AST values and 27 elevated ALT values	Average weight 1544 g (range 520-3046 g)	63 steatosis, 43 portal fibrosis, 4 cirrhosis, 11 centrolobular necrosis, 87 congestion, 52 hepato-cellular cholestasis	None	NR	NR	All hospit-alized	No search
Del Nonno *et al*[23], 2021	Italy	3	69, 63-76	M 2, F 1	Complete	NR	Admission AST: 63 (31-128) and ALT: 41 (19-84)	NR	All cases showed steatosis, portal inflam-mation, portal fibrosis, focal lobular inflam-mation, zonal necrosis and congestion	IHC: CD8+ in portal inflam-mation, CD34 positive staining in the portal tract vasculature and sinusoids, Perl's staining for iron demonstrate-d iron deposits into hepatocytes	All respiratory failure in COVID-19	1 NR, one with immunosup-pressor (tocilizumab) and one with antibiotics + morphine. All had O_2 therapy (1 CPAP and 2 venturi mask)	All hospit-alized	Negative PCR-test and IHC detection (nucleo-capsid and nucleo-protein)
Edler *et al* [24], 2020	Germany	80	79, 52-96	M 46, F 34	Complete	6 obesity, 4 cirrhosis	NR	NR	Congestion	None	76 respiratory failure in COVID-19, 1 pericardial tamponade, 1 sepsis and 2 cardiovascula-r failure	17 with NIV	51 hospit-alized, 13 in nursing care homes, 12 home deaths, 1 in a hotel and 3 NR	No search
Elsoukkary *et al*[25], 2020	United States	32	68, 30-100	M 22, F 10	Partial	17 hypertension, 12 obesity	AST: 567 (18-6000) and ALT: 387 (12-4885)	NR	9 steatosis, 6 portal inflam-mation, 3 bridging fibrosis and/or cirrhosis	None	NR	19 hydroxy-chloroquine and antibiotics, 9 only antibiotics	All hospit-alized	No search
Evert *et al*	Germany	8	62, 44-73	M 4, F 4	Complete	7 obesity, 1	NR	NR	7 cholestasis,	None	8 MOF	All did NIV,	All hospit-	3 positive

[26], 2021						liver cirrhosis, 5 hypertension			7 single-cell necrosis, 5 fatty degeneration with 2 showing marked steatosis, 2 mild fibrosis, 1 cirrhosis			dialysis and antibiotics. 5 had ECMO	alized	PCR-test
Falasca *et al* [27], 2020	Italy	22	68, 27-92	M 15, F 7	Complete	1 obesity	NR	Congestion	11 inflam-mation, 10 congestion, 12 steatosis	None	All respiratory failure in COVID-19	NR	All hospit-alized	No search
Fassan *et al* [28], 2020	Italy	26	82, 61-97	M 14, F 11	Complete	5 obesity, 1 HCV-related cirrhosis	NR	NR	1 cirrhosis, 22 congestion, 5 centrilobular parenchymal atrophy, 2 fibrosis, 5 sinusoidal diffuse microthromb-i, 3 portal vein thrombosis, 2 centroacinar necrosis, 26 activation of Kupffer cells, 1 portal inflam-mation, 9 steatosis	None	NR	NR	NR	Negative ISH
Greuel *et al* [29], 2021	Germany	6	35, 26-46	M 3 F 3	Complete	1 obesity, 2 right cardiac insufficiency, 1 Ewing sarcoma	4 elevated AST and ALT values	NR	1 severe cholestasis, 1 focal ischemic damage 2 steatosis	None	3 MOF, 1 acute mesenteric ischemia, 1 cardiovascula-r failure, 1 hemorrhagic shock	5 had ECMO and NIV	All hospit-alized	Negative PCR-test
Grosse *et al* [30], 2020	Austria	14	82, 55-94	M 9, F 5	Complete	1 liver cirrhosis, 8 hypertension	Admission AST: 49 (12-98) and ALT: 25 (7-87)	NR	13 steatosis, 14 congestion, 12 portal lymphoid infiltration, 4 portal fibrosis	None	2 bronchopneu-monia, 12 NR	12 had antibiotics	All hospit-alized	No search
Hanley *et al*	United	10	73, 52-79	M 7, F 3	Complete	5 obesity, 4	NR	Average	7 steatosis, 3	None	NR	4 NIV	All hospit-	3 positive

[31], 2020	Kingdom					hypertension		weight 1432 g (range 1012-2466) and 3 hepato-megaly	cirrhosis or bridging fibrosis				alized	PCR-test (e gene)
Hirayama *et al*[32], 2021	United Kingdom	19	71, 42-94	M 11, F 8	Complete	5 obesity, 8 hypertension	NR	NR	12 steatosis, 5 congestion, 4 cirrhosis, 3 portal inflammation	None	NR	NR	All hospit-alized	No search
Hooper *et al* [33], 2021	United States-Brazil	135	61	M 80, F 55	36 core-biopsy and 99 partial	34 obesity, 5 liver disease, 86 hypertension	NR	NR	41 necrosis, 37 steatosis, 19 inflam-mation, 7 fibrosis, 6 congestion, 5 cirrhosis, 3 cholestasis	None	101 respiratory failure in COVID-19, 6 cardiovascula -r failure, 28 NR	NR	All hospit-alized	No search
Ihlow *et al* [34], 2021	Germany	1	88	F	Complete	None	Peak AST: 1690 and ALT: 1632	Subtotal liver dystrophy	Necrosis, cirrhosis, portal inflammation	IHC for ACE2, TMPESS2 and cathepsin L: strong membranous signals in intrahepatic bile duct epithelium	Acute liver failure	Antibiotics	Hospitalized	ISH positive in the bile duct epithelium and positive PCR-test
Lacy *et al* [35], 2020	United States	1	58	F	Complete	Obesity	NR	Weight 1990 g	Steatosis and congestion	None	Respiratory failure in COVID-19	NR	Home death	No search
Lagana *et al* [36], 2020	United States	40	70, 66-80	M 28, F 12	NR	2 chronic liver disease, 1 alcohol-related cirrhosis, 1 liver transplant with acute rejection and 1 with anti-HBV core antibody positivity	*n* = 33 Admission AST: 63 (43-92) and ALT: 32 (19 - 55). Peak AST: 102 (54-294) and ALT: 68 (32-258)	2 fibrosis and 1 had abscesses, 37 with steatosis and congestion	20 lobular necroinflam-mation, 20 portal inflam-mation, 10 lobular apoptosis, 30 steatosis, 32 congestion, 16 centrilobular necrosis, 15 cholestasis	None	NR	22 steroids, 19 hydroxy-chloroquine, and 6 received tocilizumab	All hospit-alized	11 positive and 9 negative PCR-test
Lax *et al*[37], 2020	Austria	11	82, 75-91	M 8, F 3	Partial	2 obesity, 9 hypertension, 1 Hodgkin lymphoma	AST: 66 (17-189) and ALT: 41 (19-98)	NR	11 steatosis, 8 congestion, 7 necrosis, 10 Kupffer cell	None	Pulmonary arterial thrombosis	2 NIV, 9 AIRVO and 9 had antibiotics	All hospit-alized	No search

						and 1 bladder carcinoma			proliferation, 6 portal fibrosis, 8 inflammation, 8 ductular proliferation					
Malik *et al* [38], 2021	India	1	31	F	Complete	None	NR	Congestion	Congestion, mild chronic inflammatory infiltrate in some portal tract, and occasional lymphocytic aggregate adjacent to central vein	None	Respiratory failure in COVID-19	None	Hospitalized	Positive PCR-test
Menter *et al* [39], 2020	Switzerland	21	76, 53-96	M 17, F 4	17 complete and 4 partial	2 chronic liver disease, 21 hypertension, 6 obesity	*n* = 10 AST: 67.2 (22-214)	NR	7 steatosis, 5 necrosis, 3 ASH/NASH	None	Respiratory failure in COVID-19	NR	All hospitalized	No search
Nunes *et al* [40], 2021	South Africa	75	60, 49-68	M 29, F 46	Core- biopsy	41 hypertension, 20 HIV	NR	NR	33 portal inflammation, 24 steatosis, 40 sinusoidal inflammation, 10 lobular hepatitis, 9 Kupffer cell activation, 11 spotty necrosis, 4 confluent necrosis, 6 fibrosis, 26 congestion, 7 fibrin-platelet thrombi	None	NR	NR	All hospitalized	No search
Oprinca[41], 2020	Romania	3	59, 27-79	M 3	1 complete and 2 partial	1 choledochal preampular intraluminal obstruction	NR	Case 1: choledochal preampullary intraluminal obstruction, case 2: normal, case 3: hepatomegaly and cirrhosis	Case 1: congestion, steatosis, periportal fibrosis and portal inflammation, case 2: nothing, case 3:	None	2 respiratory failure in COVID-19, 1 shock hemorrhagic	Case 1: antibiotics, corticosteroids and assisted oxygenation. Case 2: none (home death). Case 3: none	2 hospitalized, 1 NR	No search

								bridging fibrosis and portal inflammation					
Rapkiewicz *et al*[42], 2020	United States	7	NR, 44-65	M 3, F 4	Complete	5 obesity and 7 hypertension	NR	NR	6 steatosis, 1 cirrhosis, 6 platelet-fibrin microthromb-i in sinusoids, 2 necrosis	None	Cardiovascular failure	5 azithro-mycina and hydroxy-chloroquine and O_2 NIV	5 hospit-alized, 2 home deaths	No search
Remmelink *et al*[43], 2020	Belgium	17	72, 62-77	M 12, F 5	Complete	2 cirrhosis, 1 liver transplant, 10 hypertension	NR	5 hepato-megaly	7 congestion, 1 steato-necrosis, 10 steatosis, 1 cholestasis, 3 chronic hepatitis, 2 cirrhosis, 1 centro-obular necrosis	None	9 respiratory failure in COVID-19, 7 MOF and 1 NR	11 had mechanical ventilation	All hospit-alized	14 positive and 3 negative PCR-test
Ren *et al*[44], 2021	China	1	53	F	Complete	None	Admission AST: 27 and ALT: 24. Peak AST: 83 and ALT: 93	Normal	Nothing remarkable	None	Respiratory failure with bacterial infection	She treated herself at home with Chinese herb medicine. In hospital intensive oxygen and supportive measurement-s, extensive antibiotics and antiviral	Hospitalized	Positive PCR-test
Schmit *et al* [45], 2020	Belgium	14	63, 50-83	M 10, F 4	Complete	1 HIV, 1 non-alcoholic steatohep-atitis, 1 HCV-hepatitis, 6 obesity	Admission AST: 54 (15-188) and ALT: 30 (7-62). Peak AST: 2610 (15-24176) and ALT: 854 (10-7245)	Average weight 1988 g (range 1280-3220 g). 8 cases yellowish appearance 6 nutmeg appearance, 2 indurated consistency, 1 hepato-cellular carcinoma, 1 normal	11 centrilobular necrosis, 9 steatosis, 8 lobular inflam-mation, 12 portal inflam-mation, 4 fibrosis, 5 bile duct proliferation, 5 cholestasis, 5 iron overload	None	13 NR and 1 acute mesenteric ischemia	8 hydroxy-chloroquine and antibiotics, 4 with antibiotics, 2 with hydroxy-chloroquine	All hospit-alized	No search
Schweitzer *et*	Switzerland	1	50	M	Complete	HIV	NR	Reduced	Steatosis and	None	Respiratory	None	Home death	No search

al[46], 2020							consistency	liver dystrophy		failure in COVID-19			
Shishido-Hara *et al* [47], 2021	Japan	1	75	M	Complete	None	NR	Normal	Portal inflammation	None	Severe hemorrhage	Anti-viral therapy, antibiotics, O_2 therapy	Hospitalized	No search
Sonzogni *et al* [48], 2020	Italy	48	71, 32-86	M 22, F 8	30 partial and 18 complete - no brain	7 obesity	47 elevated values	NR	24 lobular inflammation, 32 portal inflammation, 18 confluent necrosis 18, 26 steatosis, 48 vascular thrombosis (35 portal, 13 sinusoidal), 37 fibrosis	None	NR	NR	All hospitalized	No search
Suess *et al* [49], 2020	Switzerland	1	59	M	Complete	None	NR	NR	Steatosis and some single necrotic hepatocytes	None	Respiratory failure in COVID-19	NR	Home death	No search
Tehrani *et al* [50], 2022	Iran	5	71, 55-85	M 3, F 2	Partial	None	AST: 275 (106-528) and ALT: 392 (168-978)	NR	Congestion, hepatocytes mildly expanding and bile plugs	None	4 respiratory failure in COVID-19 and 1 cardiovascular failure	1 hydroxychloroquine and antibiotics, 2 with hydroxychloroquine and anti-viral therapy, 1 only anti-viral therapy, 1 anti-viral therapy + antibiotics	All hospitalized	No search
Tian *et al*[51], 2020	China	4	73, 59-81	M 3, F 1	Core-biopsy	1 cirrhosis and 1 hypertension	AST: 36,4 (30-48.8) and ALT: 16 (11-25.5)	NR	Case 1: congestion, glycogen accumulation and focal steatosis, case 2: regenerative nodules and fibrous bands, lobular inflammation	None	Respiratory failure in COVID-19	Antibiotics, antiviral therapy assisted oxygenation	All hospitalized	1 positive and 2 negative PCR-test, 1 was not tested

									and Kupffer cell activation, cases 3: Kupffer cell activation, case 4: periportal and centrilobular necrosis					
Varga *et al* [52], 2020	Switzerland	1	58	F	NR	Obesity and hypertension	NR	NR	Endotheliitis and necrosis	None	MOF	Dialysis	Hospitalized	No search
Wang *et al* [53], 2020	China	2	50 and 79	M 1, F 1	Core-biopsy	NR	Case 1 peak ALT and AST of 70 U/L and 111 U/L, respectively. Case 2 peak ALT and AST of 76 and 236 U/L	NR	Case 1: apoptotic hepatocytes, steatosis, lobular inflammation, portal inflammation, case 2: apoptotic bodies, steatosis, portal inflammation	IHC: case 1 increased CD68 + cells in hepatic sinusoids and infrequent CD4+. Case 2: many CD68+ cells in sinusoids	1 respiratory failure in COVID-19 and 1 septic shock	Both had antiviral therapy and antibiotics	All hospitalized	2 positive TEM (viral particles exist without membrane-bound vesicles)
Wang *et al* [54], 2020	China	1	75	F	Core-biopsy	Chronic cardiac insufficiency, hypertension	Elevated AST and ALT values	NR	Necrosis, activated histiocytes, occasional apoptotic hepatocytes, steatosis and cholestasis	None	MOF	NR	Hospitalized	Negative ISH
Xu *et al*[55], 2020	China	1	50	M	Core-biopsy	NR	NR	NR	Steatosis	None	Respiratory failure in COVID-19	Antibiotics, antiviral therapy and oxygenation	Hospitalized	No search
Yadav *et al* [56], 2022	India	21	61, 25-84	M 15, F 6	Complete	6 obesity, 1 hepatitis B, 1 multiple myeloma	Admission AST: 95.4 (18.9-760.4) and ALT: 52,1 (13,2-229,2). Peak AST: 162,6 (19,8-760,4) and ALT: 75 (21.8-229.2)	NR	20 portal inflammation, 17 steatosis, 9 lobular inflammation, 1 fibrosis, 1 vascular thrombosis, 1 necrosis	None	10 MOF, 1 multiple injuries, 6 septic shock, 3 cardiovascular failure, 1 respiratory failure in COVID-19	11 treated with antibiotics, 7 antibiotics and antiviral therapy	All hospitalized	11 positive, 9 negative PCR-test, 1 not tested

Youd *et al* [57], 2020	United Kingdom	9	72, 33-88	M 4, F 5	Complete	3 obesity	NR	4 congestion, 1 steatosis and 4 normal	NR	None	Respiratory failure in COVID-19	NR	9 deaths in community settings	No search
Zhao *et al* [58], 2020	United States	17	65, 44-85	M 10, F 7	Complete	5 hyperlipidemia, 1 cirrhosis	12 elevated AST and ALT values. Peak AST: 1903 (24-13592) and ALT 1059 (13-6136)	Weight 17694 g (1000-2600 g)	12 platelet-fibrin microthrombi, 5 histiocyte activation, 12 steatosis, 5 lobular inflammation, 8 portal inflammation, 10 necrosis	CD68 stain confirmed histiocytic hyperplasia	NR	NR	All hospitalized	5 positive IHC (spike protein) in the histiocytes in the portal tracts. Negative IHC in endothelial cells and hepatocytes

F: Female; Male: M; HCV: Hepatitis C virus; NIV: Non-invasive ventilation; ECMO: Extracorporeal membrane oxygenation; COVID-19: Coronavirus disease 2019; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ISH: *In situ* hybridization; MOF: Multi-organ failure; PCR: Polymerase chain reaction; TEM: Transmission electron microscopy; NR: Not reported; IHC: Immunohistochemistry.

to 3220 g.

Microscopic results

Microscopic results were described in 983 (99%) cases. The two most frequent findings were congestion, in 420 cases, and steatosis, in 409 cases. Four cases were described as normal. All findings are described in Table 3.

Cause of death

Cause of death was reported for 440 (44%) cases. The most frequent cause of death was respiratory failure in COVID-19, seen in 355 (81%) cases, followed by multi-organ failure in 33 cases, cardiovascular failure in 22, pulmonary thrombosis in 11, and sepsis in 8. The remaining 11 cases died respectively of hemorrhagic shock (3 cases), acute liver failure (2 cases), acute mesenteric ischemia (2 cases), bronchopneumonia (2 cases), and one case each of cardiac tamponade and multiple injuries.

Virus search

The search for the presence of SARS-CoV-2 was performed in only 162 (16%) cases. Of these 105 were tested by real-time reverse-transcription polymerase chain reaction (RT-PCR) and found positive in 53 cases, 34 cases were tested by immunohistochemistry (IHC) and all found negative, 28 were tested by *in situ* hybridization (ISH) and found negative in all cases, and lastly, 16 were tested by transmission electron microscopy and found positive in 2 cases.

Table 2 Laboratory findings

Laboratory findings	Mean (UI/L)	Range (UI/L)
Admission values (n = 53)		
AST	58	12-760
ALT	34	7-229
Peak values (n = 64)		
AST	868	15-24176
ALT	509	10-9961
Non specified		
AST (n = 61)	202	17-6000
ALT (n = 51)	209	11-4885

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

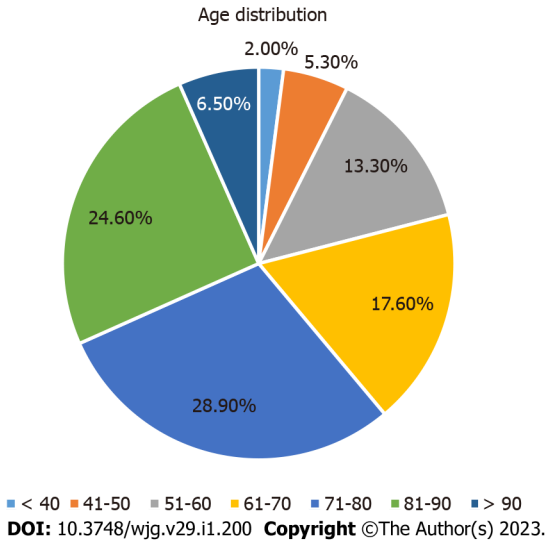
Table 3 Microscopic findings

Microscopic findings	*n* (%)
Hepatic necrosis	190 (19)
Hepatic inflammation	190 (19)
Portal inflammation	178 (18)
Fibrosis	149 (15)
Microthrombi	121 (12)
Cholestasis	114 (11)
Hemophagocytosis	51 (5)
Bile plugs	2 (0.2)
Endotheliitis	7 (0.7)
Autolysis	20 (2)
Iron overload	5 (0.5)
Other (abscess, ductal proliferation and granulomatosis)	15 (1.5)

DISCUSSION

A total of 994 autopsy cases of COVID-19 patients with liver assessment were found in the literature. As expected, more than half of the deceased were males and age distribution was highly variable, with a predominance of subjects in the age group 60-90 (71.1%).

Pre-existing liver disease was rare (6%-literature data shows a frequency of 2%-11%), with only 16.2% of the cases presenting obesity (BMI > 30 kg/m^2)[7]. Obesity, in association with diabetes and hypertension, is a prominent risk factor for severe disease and could predispose to nonalcoholic fatty liver disease (NAFLD), a metabolic syndrome which is known to suppress the pro-inflammatory M1 macrophages favoring the progression of virus infection[2,8,11]. NAFLD seems to be identified with a higher prevalence in patients with severe COVID-19 and predisposes to higher liver enzymes at admission and at discharge[59]. To date the fact that pre-existing liver disease is an independent risk factor for poor outcome is still debated; for some authors patients with liver diseases are not over-represented in hospital casuistry[4,60-62], while for others the presence of a pre-exiting illness is index of a greater probability of a bad outcome[7,63-65]. This does not count in the case of cirrhosis, seen in only 1% in this review, which is known to be an important predictor of mortality, with a mortality rate of 31%[2,61]. It appears that in the case of cirrhosis those who survive the first insult have a re-admission rate in hospital similar to those with cirrhosis, but without COVID-19, indicating that beyond the acute phase SARS-CoV-2 does not change the natural history of the disease[4]. There are currently few data regarding the mortality rate associated with alcohol liver disease as an independent risk factor, mainly related to the difficulties of correlating liver damage or elevation of liver enzymes to alcohol



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Figure 1 Age distribution.

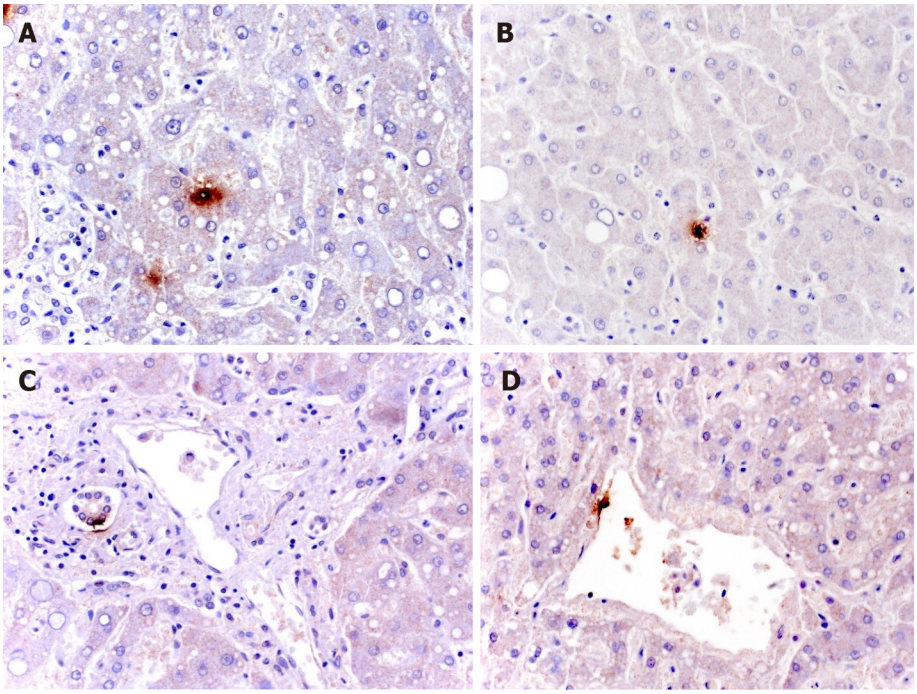
consumption. To date, it seems that alcohol liver disease increases the mortality risk by 1.8 fold[61].

Laboratory findings have not been collected in a homogeneous way, with 27 papers not reporting any data, 5 reporting AST and ALT values at admission, and 8 reporting the maximum values during hospitalization, and 4 reports for AST and 5 papers for ALT described the laboratory values without specifying the timing of the sampling. Abnormal values, without specifying the laboratory values, were described in 5 articles. From literature data it appears that liver enzyme abnormalities have a wide range, occurring in 14%-76% of the cases[4,5,7,11,66]. This great range, as Marjot *et al*[4] pointed out, could be attributed to different limits of the definition of normal values. It is still debated whether elevated liver enzymes are associated with a greater risk of mortality, because patients with worst outcomes tend to be monitored in intensive care units, while those with mild symptoms are not strictly monitored. Thus, the use of abnormal laboratory findings at admission as a predictor of poor outcome is still not sure. Liver enzyme elevation mainly affects AST and ALT, indicating hepatocellular damage rather than cholestatic, despite a greater expression of ACE2 receptor in cholangiocytes[3]. As the study of Wong *et al*[67] pointed out, the odd ratio of elevated AST and ALT levels in COVID-19 patients is 3.4 and 2.5, respectively.

Due to the presence of such fragmented laboratory data, it is difficult to draw conclusions about the trend of laboratory values during hospitalization, although some authors have found a tendency of increased values during hospitalization, in particular in those in critical conditions[9,11,12,68,69]. Whether enzyme elevation is induced directly by the virus or because of the inflammation, congestion, or medications is still not clear. Certainly, many of the drugs used in COVID-19 positive patients turn out to be hepatotoxic such as hydroxychloroquine and antivirals such as ritonavir, lopinavir, and remdesivir[8,66]. The meta-analysis by Wong *et al*[67] and Cai *et al*[66] suggests that liver injury is higher in studies with high usage of lopinavir/ritonavir, despite that their hepatotoxic role is still to be described in patients without pre-existing liver disease, while there was no evidence of a higher risk of liver injury for those treated with antibiotics, nonsteroidal anti-inflammatory drugs, ribavirin, herbal medications, and interferon.

The literature review highlighted the presence of a great discrepancy in the autopsy protocols, with only half of the autopsies performed as complete (full autopsies), while the other half as partial. Macroscopic evaluation of the liver was not frequent, while microscopic assessment was present in almost every case (99%). As expected, congestion and steatosis were the most frequent findings. The congestion can be traced back to the presence in these patients of cardiovascular dysfunction due to the massive inflammation and cytokine storm linked to the infection. The presence of steatosis needs a more complex analysis; lipid accumulation due to SARS-CoV-2 has to be differentiated from pre-existing modifications, typical of patient with metabolic syndrome. COVID-19 lipid accumulation can be explained because of the cytopathic effect of the coronavirus, which induces endoplasmatic stress and lipogenesis[2]. Transcriptomic profiling of COVID-19 patients by Wanner *et al*[5] demonstrated an upregulation of cellular processes involved in lipid/cholesterol synthesis. Furthermore, corticosteroid therapy, widely used in the treatment of COVID-19, is known to be associated with steatosis or glycogenosis[2].

Hepatic necrosis and inflammation can be multifactorial; they can be induced by a cytopathic direct effect of the virus, because of inflammatory storm or hypoxic hepatitis, or may be drug induced. These hepatic changes are the third most frequent finding in liver autopsies of COVID-19 patients[70]. Differentiating the different causes from a pathological point of view is impossible, also in consideration of



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Figure 2 Immunohistochemical staining for severe acute respiratory syndrome coronavirus 2 in liver tissue. A and B: Spot localization of virus in samples of initial hepatic necrosis and in Kupffer cells; C and D: Spot localization of isolated ductular and endothelial cells (mouse, GeneTex GTX632604, 1A9 clone, 1:100).

the fact that they can overlap one another. In addition, patients with pre-existing liver diseases, such as chronic liver disease, have an increased risk of drug-induced hepatic damage, therefore in those patients the use of hepatotoxic treatments should be weighted. Liver damage in critically ill patients is known and is linked to the so-called hypoxic hepatitis, which is caused by underlying cardiac dysfunction and respiratory failure that decrease the blood flow and oxygenation inducing cellular stress. Moreover, damage could even be mediated by reperfusion, which promotes the production of reactive oxygen species, leading to damage. This process can be highlighted in some cases as a picture of endotheliitis[2,3,11]. Massive inflammation is common in COVID-19 patients and macrophage activation is evidenced by the presence of hemophagocytosis in liver tissue.

Unlike what is reported by Marjot *et al*[4], the frequency of thrombotic phenomena of the hepatic vascular tree is lower, with 12% of cases instead of 29%. As Kleiner[70] noted, death could occur long after the acute phase of liver damage, so the histological changes do not always represent a reliable image of what happened in acute damage, but are the result of damage and reparative modifications. Therefore, to better understand the acute damage, it could be of help to perform a liver biopsy in patients with liver damage. Obviously, it is understood that the execution of such an invasive examination is not a priority in the treatment of these patients, but it could be performed in those cases where the hepatic injury dominates the clinical picture.

Despite the presence of hepatic injury, the presence of SARS-CoV-2 in the liver has been sought infrequently (16% of the cases). Most studies have exploited the RT-PCR to search for the viral genome, but only a few have applied other techniques (IHC, ISH, and transmission electron microscopy) to identify the cells in which the viral proteins were expressed (Figure 2A and B). It is not surprising that by using RT-PCR a greater number of cases resulted positive, because this type of analysis uses a homogenized tissue, which also contains vessels and immunity cells. However, the few available data allow us to confirm the fact that the virus can be found mainly in Kupffer cells, endothelial cells of centrolobulare veins, and cholangiocytes (Figure 2C and D). Note that Wanner *et al*[5] demonstrated that, when comparing the levels of SARS-CoV-2 RNA copies per cell between airway samples and autopsy livers biopsies, the levels of RNA show similar ranges, but with lower median RNA in liver specimens.

CONCLUSION

Postmortem investigations remain the gold standard to investigate the effects of SARS-CoV-2 in different organs and apparatuses. It is well known that the absence of postmortem investigations in the first wave of the pandemic has failed to provide a valuable contribution to the correct management and

treatment of patients. On the other hand, the execution of clinical and forensic autopsies has disclosed several important aspects of the disease, clarifying morphological and virologic features and promoting unexplored therapeutic approaches and new frontiers of research[71-74]. Despite the limited number of performed autopsies worldwide, to date there is no doubt that the liver is a target for the virus, despite minimal viral receptor expression. However, liver damage is not always directly linked to the action of the virus, but can be secondary to inflammation or even simply caused by the therapy administered during hospitalization. Therefore, it is important to monitor patients who use hepatotoxic drugs, to avoid worsening of the liver functions, which can affect the patient's outcome.

ARTICLE HIGHLIGHTS

Research background

Hepatic histologic manifestations of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection are far to be completely investigated. Many authors demonstrated the presence of angiotensin converting enzyme 2 receptor in the liver as well as transmembrane serine protease 2.

Research motivation

Liver injury was demonstrated in 14%-53% of cases of patients with SARS-CoV-2 infection. In the first wave of the pandemic few autopsies were performed and only few authors can provide a wide casistic. Authors started to study the histologic manifestations of coronavirus disease 2019 (COVID-19) in the lungs, heart, and liver, too.

Research objectives

The objectives of the study were to summarize the biochemical and histological changes in the liver and to promote the leading role of autopsy in the pandemic.

Research methods

Authors provide a systematic review focusing on autopsy studies of COVID-19 deaths and in particular on liver pathology.

Research results

Forty-six articles corresponding to the inclusion criteria were included, with only 994 autopsy cases of COVID-19 patients. Congestion and steatosis were the main histopathological findings, followed by hepatic necrosis, hepatic and portal inflammation, and fibrosis. The most frequent cause of death was respiratory failure, pulmonary thrombosis, and sepsis. Acute liver failure was indicated as the cause of death in two cases.

Research conclusions

The review of the literature highlighted the presence of a great discrepancy in the autopsy protocols, with only half of the autopsies performed as complete (full autopsies), while the other half as partial. Macroscopic and microscopic evaluation of the liver was not always performed or described. Despite the presence of hepatic injury, the presence of SARS-CoV-2 in the liver has been sought infrequently (16% of the cases).

Research perspectives

Much more effort needs to be addressed to completely investigate liver toxicity from COVID-19. Autopsies had a leading role during the pandemic and were important to understand the physiopathology of SARS-CoV-2 infection and should be always considered to improve scientific research.

FOOTNOTES

Author contributions: Zanon M and D'Errico S contributed to the writing and conceptualization; Neri M and Pizzolitto S contributed to the formal analysis and investigation; Radaelli D and Concato M contributed to the data curation; Peruch M contributed to the supervision.

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PRISMA 2009 Checklist statement: The review followed the PRISMA 2009 checklist statement.

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Country/Territory of origin: Italy

ORCID number: Martina Zanon 0000-0002-8970-9133; Margherita Neri 0000-0002-6459-1669; Davide Radaelli 0000-0002-7053-6463; Monica Concato 0000-0003-1026-3106; Michela Peruch 0000-0001-8863-7126; Stefano D'Errico 0000-0003-3689-0001.

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RETRACTION NOTE

Retraction note to: Beneficial effect of probiotics supplements in reflux esophagitis treated with esomeprazole: A randomized controlled trial

Qing-Hua Sun, Hong-Yan Wang, Shi-Dong Sun, Xin Zhang, Han Zhang

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Qing-Hua Sun, Department of Gastroenterology, Peking University Third Hospital, Beijing 100191, China

Hong-Yan Wang, Shi-Dong Sun, Xin Zhang, Han Zhang, Department of Gastroenterology, PKUCare Luzhong Hospital, Zibo 255400, Shandong Province, China

Corresponding author: Shi-Dong Sun, MS, Doctor, Department of Gastroenterology, PKUCare Luzhong Hospital, No. 65 Tai Gong Road, Linzi District, Zibo 255400, Shandong Province, China. sunshidong156@163.com

Abstract

We have decided to retract the above article for further consideration due to a data labelling error.

Key Words: Retraction note

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Core Tip: We have decided to retract the above article due to a data labelling error.

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RETRACTION NOTE

Retraction Note to: Beneficial effect of probiotics supplements in reflux esophagitis treated with esomeprazole: A randomized controlled trial. *World J Gastroenterol* 2019; 25: 2110-2121 [PMID: 31114137 DOI: 10.3748/wjg.v25.i17.2110]. The online version of the original article can be found at https://www.wjgnet.com/1007-9327/full/v

25/i17/2110.htm.

It has come to my attention that there was a data labelling error in the study[1] we published in the *World Journal of Gastroenterology* four years ago. We discovered that one of our research assistants confused the two drugs by mistake when dispensing them to subjects, meaning that the labels for patients may have been mismatched with the actual treatment they received. Due to this data labelling problem, the validity of the paper and its conclusion have been brought into question even though the methodology of statistical analysis used in the paper was correct. To avoid misleading other readers, I respectfully ask that the article "Beneficial effect of probiotics supplements in reflux esophagitis treated with esomeprazole: A randomized controlled trial. *World J Gastroenterol* 2019; 25: 2110-2121 [PMID: 31114137 DOI: 10.3748/wjg.v25.i17.2110]" is retracted.

We apologize to the readers and editors of the *World Journal of Gastroenterology* for this error and for any inconvenience caused.

FOOTNOTES

Author contributions: Sun QH, Wang HY, Sun SD, Zhang X, Zhang H wrote this retraction note.

Conflict-of-interest statement: The authors declare that there are no conflicts of interest.

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Country/Territory of origin: China

ORCID number: Qing-Hua Sun 0000-0001-5029-587X; Hong-Yan Wang 0000-0002-6837-5146; Shi-Dong Sun 0000-0002-8945-9170; Xin Zhang 0000-0001-9514-1292; Han Zhang 0000-0002-3028-5460.

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Editorial Board Member of *World Journal of Gastroenterology*, Satoshi Ono, PhD, Director, Department of Gastroenterology and Gastrointestinal Endoscopy, Tokyo Metropolitan Geriatric Medical Center, 35-2, Sakae-Cho, Itabashi, Tokyo 173-0015, Japan. satoshi-tky@umin.ac.jp

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OPINION REVIEW

Irreversible electroporation for the management of pancreatic cancer: Current data and future directions

Stavros Spiliopoulos, Lazaros Reppas, Dimitrios Filippiadis, Antonella Delvecchio, Maria Conticchio, Riccardo Memeo, Riccardo Inchingolo

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Stavros Spiliopoulos, Lazaros Reppas, Dimitrios Filippiadis, Second Department of Radiology, School of Medicine, National and Kapodistrian University of Athens, Athens 12461, Attiki, Greece

Antonella Delvecchio, Maria Conticchio, Riccardo Memeo, Unit of Hepato-Pancreatic-Biliary Surgery, "F. Miulli" Regional General Hospital, Acquaviva delle Fonti 70021, Bari, Italy

Riccardo Inchingolo, Interventional Radiology Unit, "F. Miulli" Regional General Hospital, Acquaviva delle Fonti 70021, Italy

Corresponding author: Stavros Spiliopoulos, MD, PhD, Associate Professor, Second Department of Radiology, School of Medicine, National and Kapodistrian University of Athens, 1st Rimini Street, Athens 12461, Attiki, Greece. stavspiliop@med.uoa.gr

Abstract

Pancreatic cancer is currently the seventh leading cause of cancer death (4.5% of all cancer deaths) while 80%-90% of the patients suffer from unresectable disease at the time of diagnosis. Prognosis remains poor, with a mean survival up to 15 mo following systemic chemotherapy. Loco-regional thermal ablative techniques are rarely implemented due to the increased risk of thermal injury to the adjacent structures, which can lead to severe adverse events. Irreversible electroporation, a promising novel non-thermal ablative modality, has been recently introduced in clinical practice for the management of inoperable pancreatic cancer as a safer and more effective loco-regional treatment option. Experimental and initial clinical data are optimistic. This review will focus on the basic principles of IRE technology, currently available data, and future directions.

Key Words: Pancreatic cancer; Interventional oncology; Irreversible electroporation; Ablation; Loco-regional treatment; Image-guided treatment

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Core Tip: Loco-regional thermal ablative techniques such as radiofrequency, microwave, and cryoablation are rarely implemented for the treatment of inoperable pancreatic cancer due to the increased risk of thermal injury to the adjacent structures. Irreversible electroporation is a promising novel non-thermal ablative modality that could provide a safer and effective ablation *via* the application of electric pulses to damage cell membranes and cell homeostasis resulting in cancer cell necrosis and apoptosis. Experimental and initial clinical data are optimistic, and its potential immunomodulatory effect and synergism with immunotherapy provides are promising.

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INTRODUCTION

Pancreatic cancer is currently the seventh leading cause of cancer death, representing 4.5% of all cancer deaths worldwide. Most importantly, overall prognosis remains extremely poor as approximately 80%-90% of patients suffer from unresectable disease at the time of diagnosis, with a less than 8% relative survival rate at 5 years[1]. Systemic chemotherapy using gemcitabine or more recently FOLFIRINOX regimens, with or without radiotherapy, results in overall survival rates ranging from 9 to 14 mo[2,3]. Moreover, thermal (radiofrequency and microwave) and cryoablative techniques are not commonly employed due to the increased risk of severe trauma to the adjacent major anatomical structures[4]. Irreversible electroporation (IRE) is a promising novel percutaneous, image-guided nonthermal ablative modality that has been recently introduced in clinical practice for the management of pancreatic cancer.

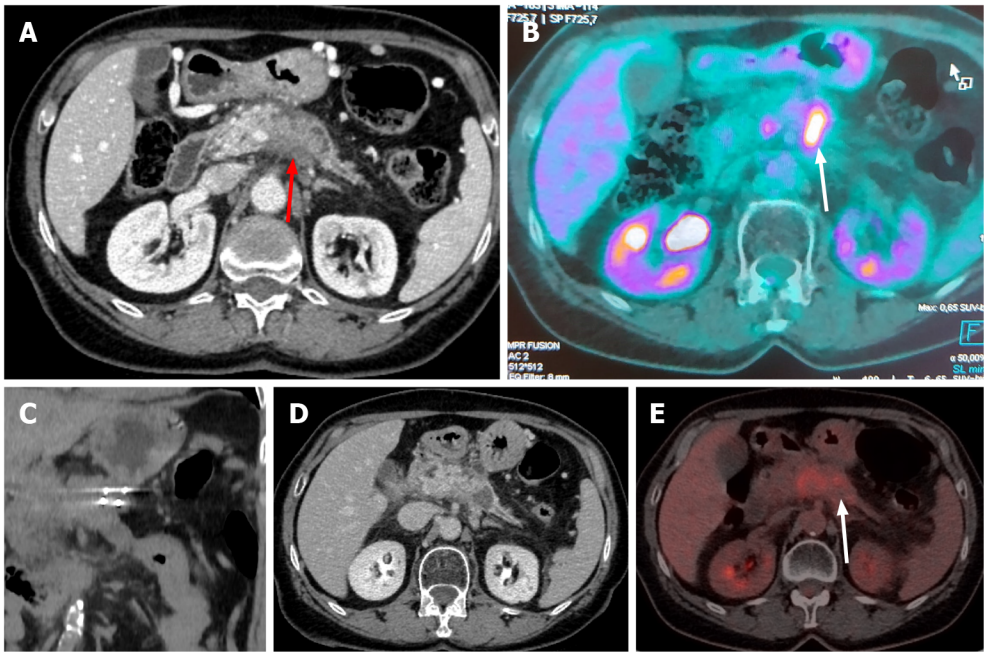
MECHANISM OF ACTION

The phenomenon of IRE was first reported in the 1970s to describe the alteration of transmembrane potential leading to the increased cell membrane permeability, disruption of the dual lipid layer, and the creation of permanent nanoscale defects (nanopores) in the cell membrane following the application of high-voltage pulsed electric fields across the cell[5]. This technique results in failure of the cell homeostasis, electrolyte alteration, and cell death by apoptosis[6-9].

In contrast to necrosis induced by thermal ablative methods, non-thermal apoptotic active cell death does not incite inflammation and enables ablation with minimal distortion of the adjacent tissues. However, since 2006 when the first *in vivo* model of IRE for cancer ablation was reported, several experimental studies have reported solely necrosis or mixed results of both necrosis and apoptosis following the application of IRE[10-13]. According to currently available experimental data, apoptosis has been demonstrated immediately after application of IRE in a murine cancerous pancreas model and at 7 to 14 d in a porcine healthy pancreas model, while necrosis is evident immediately and up to 14 d later. Unfortunately, pathology data on human pancreatic cancer are extremely limited and as the IRE ablative effect is directly correlated to the physical properties of the target tissue, the significant discrepancies between *in vivo* normal/cancer animal models, and human cancer/normal pancreatic tissues. This presents a major limitation regarding our knowledge on the actual effects of IRE[14-16]. Moreover, data indicate that IRE is not homogenously distributed along the target tissue, and various effects are produced with increasing voltage and time.

While apoptosis is certainly occurring in some cells within the treatment zone, Brock *et al*[17] suggest that IRE could initiate multiple types of cell death mechanisms, but the size and shape of the regions in which each type is experienced may vary between clinical treatments depending on differences in pulsing parameters, tissue type, and treatment time. Thus, there may be more than one type of cell death mechanism at play, and may include pyroptosis or necroptosis. Likewise, for cells at the margins of the treatment areas, the response observed may actually be survival signaling in response to reversible electroporation. In theory, this could be taken advantage of and combined with chemotherapy treatments to increase drug delivery, tumor penetration, and treatment of residual cancer cells[17,18].

IRE has significant inherent advantages over thermal ablation for the treatment of pancreatic cancer (Figure 1). Most importantly, IRE does not produce a temperature increase to achieve tumor destruction. Therefore, it does not elicit thermal injury to the superior mesenteric and portal veins, superior mesenteric and celiac arteries, bile duct adjacent nerves, or gastrointestinal structures, which has restrained the use of local thermal ablation treatment. Another significant advantage is the absence of



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Figure 1 Pancreatic cancer treated with percutaneous irreversible electroporation. A: Axial computed tomography (CT) showing a lesion (arrow) in the body of the pancreas consistent with ductal adenocarcinoma previously treated with chemo and radiation therapy; B: Positron emission tomography (PET)-CT scan showed the residual active part of the lesion (arrow); C: CT coronal view with 4 parallel electrodes positioned within the lesion; D and E: 3-mo follow-up CT (D) and PET-CT (E) showing complete ablation of the tumor with photopenic area in the site of ablation (arrow).

the "heat sink" effect in which the flow of large blood vessels decreases the thermal ablative effect[19-22]. According to published clinical protocols, the procedure is performed under computed tomography (CT) guidance, general anesthesia (complete muscle paralysis), and electrocardiography synchronization due to the possibility of muscular spasms induced by high-voltage pulses[23].

IRE is induced by electrodes connected to a high-voltage pulse generator. Multiple electrode pairs can be used; the number of electrodes needed and their exact placement is decided during pre-procedural planning. For small tumors measuring up to 2 cm, three electrodes are placed at the periphery of the target lesion, and for lesions between 2 cm and 3 cm, four electrodes are used. However, for lesions > 3 cm, a maximum number of six electrodes is allowed with four or five electrodes at the periphery and one or two at the center of the lesion. An optimal interelectrode distance between 7 mm and 24 mm has been described. The correct positioning of electrodes requires experience often deriving from that with other ablative methods. Skill is also needed in using ultrasound or CT techniques as a guide for positioning the electrodes and avoiding accidental damage to surrounding organs.

With respect to large vessels close to the tumor, a minimum safety distance of 2 mm is recommended to avoid the risk of burn damage. In cases of locally advanced pancreatic cancer with involvement of the mesenteric artery or vein, placing the electrodes parallel to the vessels has proven effective. Following electrode placement, the generator produces short, repeated pulses using predetermined voltage settings to reach a target current of 20-50 A. In clinical practice usually, 90 pulses per treatment cycle are used, with a pulse duration of 70-90 μs, and a voltage setting between 1400-1800 V/cm (maximum capability 3000 V/cm)[2,9,24-26]. According to standard ablation technique protocols, the aim is to create a safe 5 mm tumor-free IRE zone, also referred as A0 ablation (analogous to an R0 surgical resection).

PRE-CLINICAL DATA

To date, only a few reports on IRE for the treatment of pancreatic cancer exist in literature. Some of them reported the use of the technique on animal models (Table 1). These studies have used animal xenografts carrying human pancreatic cancer cells to understand the histological effect of IRE on pancreatic cancer tissue. The results following application of IRE showed evidence of both acute coagulative necrosis and apoptosis of pancreatic cancer tissue followed by fibrosis.

In 2010, Charpentier *et al*[27] reported a pilot study on IRE in a normal pancreas porcine model. They showed the following histological features: Initial active local inflammation, interstitial edema, and significant necrosis (after 7 d) followed by the development of fibrosis. However, these results were not significant for IRE efficacy because normal pancreatic tissues had a very different conductivity than

Table 1 Irreversible electroporation of pancreatic cancer in animal models

Ref.	Animal model	*n*	Tumour location	Histology	Major complication
Charpentier *et al*[27], 2010	Porcine	4	NP	Necrosis	None
Bower *et al*[28], 2011	Porcine	6	Orthotopic PC	Necrosis	None
Joséet al[29], 2012	Mouse	24	Orthotopic PC	Necrosis	None
Fritz *et al*[33], 2015	Porcine	10	Orthotopic PC	Necrosis	None
Su *et al*[30], 2018	Mouse	22	Orthotopic PC	Necrosis	None
Shankara Narayanan *et al*[31], 2018	Mouse	N/A	Subcutaneous/orthotopic PC	Necrosis	None
Lee *et al*[32], 2021	Porcine	N/A	Orthotopic PC	Necrosis	None

N/A: Not available; NP: Normal pancreas; PC: Pancreatic cancer.

pancreatic tumors.

Subsequently, Bower *et al*[28] and José *et al*[29] suggested that IRE could be an effective treatment for locally advanced pancreatic cancer in a mouse model without systemic toxicity and major local complication. Su *et al*[30] concluded that IRE was a safer and shorter operation than traditional ablation and represented a promising new approach for pancreatic cancer. Narayanan *et al*[31] described IRE for a pancreatic cancer mouse model and concluded that this animal model serves as a robust system to study the effects and clinical efficacy of IRE. Also, Lee *et al*[32] demonstrated and confirmed on a porcine model the safety and minimal complications of IRE ablation in pancreatic cancer tissue. Finally, the results of IRE in animal models of the treatment of pancreatic cancer showed the ability to ablate the target cells while preserving the collagen architecture of vascular, biliary, and neuronal structures[12, 28].

CLINICAL DATA

The prognosis of patients with pancreatic cancer not eligible for surgery remains poor despite many chemoradiation protocols. Therefore, different approaches for treatment of this disease are required. Ablation procedures including radiofrequency ablation, microwave ablation, cryoablation, high intensity focused ultrasound, and IRE can offer symptomatic relief, survival benefit, and potential tumor downsizing. Nevertheless, thermal procedures using extreme temperatures can induce injury to the pancreatic duct, bile duct, and adjacent vessels, potentially resulting in fistula formation, bile leaks, and hemorrhage, respectively[34].

IRE is an emerging non-thermal local ablation technique that affects with electricity only target cell membranes and avoids the nearby vessels and vital structures. Therefore, IRE can also be used in tumors positioned near some vital structures or organs more safely compared to other ablative procedures[35]. According to the American Joint Committee on Cancer stage criteria (8th edition), the major current indications for the use of IRE in the treatment of pancreatic cancer are as follows: Locally advanced pancreatic cancer stage II or III (T4N1M0) with ≤ 3 positive regional lymph nodes; tumor size maximal axial diameter ≤ 5 cm; and tumors in patients not candidates for radical resection or who refuse this surgery. IRE also carries some absolute and relative contraindications. It cannot be used if there are metal implants less than 2.5 cm from the ablation area, or in patients with portal vein occlusion, portal hypertension, ascites, bile duct obstruction, or hyperbilirubinemia. Additionally, IRE can also affect myocardial contraction mechanisms and as such cannot be applied in patients with cardiac arrhythmias, previous heart failure, active coronary disease, or recent pacemaker implantation. Finally, IRE cannot be used in patients with epilepsy despite the fact that it not been proven to cause brain stimulation[35].

The indications and contraindications for IRE for the treatment of pancreatic cancer are summarized in Table 2. Martin *et al*[36] and Narayanan *et al*[37] described the first clinical series on the implementation of IRE for the treatment of human pancreatic cancer. Since then, the use of this technique has been widespread[38-40], but to date there is still no defined protocol for the use of IRE in the treatment of pancreatic cancer. Studies showed that IRE was a viable treatment for locally advanced pancreatic cancer or borderline resectable pancreatic cancer because it allowed tumor downstaging, definitive locoregional treatment, or adjuvant treatment following resection[41-43]. In human tumor tissue, IRE induces necrosis as it does in animal cancer models; however, there is no evidence of apoptosis[16]. A series of retrospective and prospective clinical studies on human pancreatic cancer treated with IRE suggested a survival benefit with a median overall survival (OS) up to 30 mo[38,39].

Combined treatments involving IRE, chemotherapy, and immunotherapy can offer a multimodal approach which can limit the disease progression. However, the debate is ongoing with respect to the

Table 2 Indication and contraindications of irreversible electroporation in pancreatic cancer

Indication	Contraindications	
	Absolute	Relative
Biopsy-proven primary and solitary pancreatic tumors	History of ventricular arrhythmias	Atrial fibrillation
Locally advanced pancreatic cancer. Stage II or III (T4N1M0) with regional positive lymph nodes ≤ 3	Implanted cardiac stimulation devices within 1 yr	Coronary artery disease
Tumor size maximal axial diameter ≤ 5 cm	Uncontrollable hypertension	Combined severe stenosis of the common hepatic artery and main portal vein branch
Patients not candidates for radical resection or who refuse the surgical operation	History of epilepsy	Metallic foreign object in the ablation zone
Patients with a predictable OS longer than 3 mo, Karnofsky Performance Score > 50	Congestive heart failure (> NHYA class 2)	Liver failure, portal hypertension, ascites, bile duct obstruction, hyperbilirubinemia
		Irreversible bleeding disorders
		Uncontrolled infections; patients that have received chemo or immunotherapy in the 4 wk prior to treatment

OS: Overall survival; NHYA: New York Heart Association.

timing of multimodal treatment. Some studies that administered IRE before chemotherapy showed only a modest increase in survival; Månsson *et al*[40] reported a median OS of 13 mo. In contrast, studies using IRE after induction chemotherapy reported an increase in survival with a median OS of 27 mo [44]. Despite improvements in radiation therapy, chemotherapy, and surgical procedures over the last 30 years, pancreatic cancer 5-year survival rate remains at 9%.

Recently, the advanced techniques of proton radiation and carbon ion radiation therapies have been used for locally advanced pancreatic cancer with encouraging results. The proton beam offers significant physical advantages over the photon due to the Bragg peak effect with little or no output dose beyond the tumor target, thereby sparing any critical organs adjacent to cancer. Compared to proton radiation, carbon ion radiation offers similar dosimetric characteristics, but it has a substantially different biological property and offers greater biological efficacy in inducing complex DNA damage, leading to an increase in the destruction of cancer cells[44].

Despite the non-thermal effect of IRE, complications related to the production of heat near the electrodes (defined as secondary Joule heating) remain unavoidable[45]. The most common complications following IRE are mild acute pancreatitis, pain, diarrhea, nausea, vomiting, loss of appetite, and delayed gastric emptying. Serious complications after IRE related to the location and size of the pancreatic tumor have also been reported in the literature and include arrhythmia, severe acute pancreatitis, hemorrhage, portal vein thrombosis, bile or pancreatic fistula, gastrointestinal tract perforation, and death[16,45]. In one of the most recent reviews[16], the average rate of serious complications after IRE was 12%, with a maximum reported value of 42%[2]. The size of the tumor is one of the most important factors related to procedure complications; for example, Narayanan *et al*[46] treated patients with tumors up to 8 cm in size and reported one of the highest total complication rates of 62%. Other factors contributing to post IRE complications depend on the team experience, the protocol used, and the type of approach (open *vs* percutaneous)[18]. For example, the average mortality rates have been reported as 2% and 0% for open and percutaneous IRE, respectively[20].

Available IRE protocols in part derive from data gathered from animal studies; however, the pancreas tissue of animals and humans differ significantly in cellular composition and electrical impedance. In addition, available IRE protocols developed to date differ in recommended distance between the electrodes and intensity of applied voltage. Moreover, these protocols vary in the reported individual electrical properties of the tissue being ablated, which can have an impact on the effectiveness of the treatment and on the area of ablation itself[16]. This variation highlights an important knowledge gap, which can be attributed in part to the risks and ethics of *in vivo* human tissue sampling. One way to bridge this gap is to apply IRE to both diseased and healthy perfused human organs. Use of IRE on *ex vivo* perfused pancreas, for example, could help to shape a treatment protocol for the use of IRE in the treatment of pancreatic cancer[16]. Indeed, IRE is not yet widely used in clinical practice because there is a lack of consensus on the optimal IRE treatment protocol and for the approach required to protect adjacent pancreatic tissue[25]. Evaluation of the benefits following IRE are needed in pancreatic tumor tissue in order to establish these appropriate treatment protocols.

Future directions

The main issue surrounding the use of IRE for the treatment of pancreatic cancer is the absence of randomized data. Currently, two major randomized controlled trials (RCT) are recruiting patients in order to provide level 1 safety and efficacy evidence *vs* standard of care. The PAL-PIE study is a United Kingdom-based multicenter RCT that will recruit 50 patients (from up to seven pancreas centers) with locally advanced pancreatic cancer and previous first-line chemotherapy (5-fluorouracil, leucovorin, irinotecan and oxaliplatin) randomized to treatment with IRE (plus chemotherapy if indicated) *vs* chemotherapy alone[47]. The DIRECT study is a randomized, multicenter, controlled, unblinded study to assess the safety and efficacy of the NanoKnife® system for the ablation of unresectable stage III pancreatic adenocarcinoma. This study will randomize over 500 patients with stage III pancreatic cancer to receive IRE plus chemotherapy (a modified FOLFIRINOX regimen) *vs* chemotherapy alone; the estimated completion date is December 2023 (ClinicalTrials.gov Identifier: NCT03899636).

Additionally, following positive initial clinical outcomes, researchers are currently investigating the interesting concept of IRE-induced immune response to cancer[48-51]. IRE has been identified as a potential immunomodulatory therapy due to post-ablation release of intracellular tumoral antigens that act as *in situ* immunization agents resulting in both local and systemic response to remaining cancer cells. Specifically, IRE can remodel the local tumor microenvironment by smoothing the extracellular matrix, alleviating hypoxia, and assisting in the infiltration of immune cells into residual cancer foci. Moreover, the combination of IRE and immunotherapy could incite potent synergistic antitumoral effects[24]. Nevertheless, the mechanisms involved in immunomodulation following IRE in humans remains unclear[52]. However, trials focusing on the potential of IRE combined with immunotherapy to improve prognosis of unresectable pancreatic cancer are ongoing. For example, a pilot multicenter, single-arm phase II trial is currently recruiting patients with metastatic pancreatic ductal adenocarcinoma to investigate whether the combination of IRE treatment of one liver metastasis followed by nivolumab treatment leads to a measurable radiological response (ClinicalTrials.gov Identifier: NCT04212026).

Combination therapy of IRE with chemotherapeutic regiments is also being evaluated as the rim of peripheral sensitivity to chemotherapy produced around central tumor necrosis following IRE typically presents as microscopic peripheral seeding[53]. To this end, several ongoing prospective trials such as the CHEMOFIRE-2 trial (Chemotherapy Followed by Irreversible Electroporation in Patients With Unresectable Locally Advanced Pancreatic Cancer; ClinicalTrials.gov Identifier: NCT04093141) are underway.

Improvements with respect to IRE technology itself are also needed. An interesting technique requiring further investigation is endoscopic IRE, which could provide a solution for patients without safe transabdominal access. A major limitation of IRE is the intent of producing a small ablation zone of approximately 1-1.5 cm, which requires several electrodes to produce the desired A0 ablation. This requirement renders the procedure more technically demanding and time-consuming compared to conventional thermal ablation modalities[54]. Future research should focus on the standardization and optimization of an IRE treatment protocol for the treatment of pancreatic cancer with the goal of providing maximum efficacy without damaging surrounding tissues. It should also aim to refine the parameters of post-treatment radiological assessment for the development of objective and measurable predictors of treatment outcomes following use of IRE.

CONCLUSION

As demonstrated by initial preclinical and clinical data, the unique characteristics of IRE render this non-thermal ablation modality most suitable for the minimally invasive treatment of locally advanced pancreatic cancer. The synergic effect of IRE combined with chemo- or immunotherapy could significantly improve outcomes. Further investigation is required to elucidate its exact mechanism of action, optimize treatment protocols, and provide high-quality comparative clinical data for the management of patients with pancreatic cancer.

FOOTNOTES

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Country/Territory of origin: Greece

ORCID number: Stavros Spiliopoulos 0000-0003-1860-0568; Antonella Delvecchio 0000-0002-7759-4340; Maria Conticchio 0000-0003-3177-5274; Riccardo Inchingolo 0000-0002-0253-5936.

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OPINION REVIEW

Acute-on-chronic liver failure: Controversies and consensus

Natalie L Y Ngu, Eliza Flanagan, Sally Bell, Suong T Le

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Natalie L Y Ngu, Eliza Flanagan, Sally Bell, Suong T Le, Department of Gastroenterology and Hepatology, Monash Health, Clayton 3168, Victoria, Australia

Natalie L Y Ngu, Eliza Flanagan, Sally Bell, Suong T Le, Faculty of Medicine, Nursing, and Health Sciences, Monash University, Clayton 3800, Victoria, Australia

Natalie L Y Ngu, Department of Gastroenterology and Hepatology, Alfred Health, Melbourne 3004, Victoria, Australia

Eliza Flanagan, Suong T Le, Monash digital Therapeutics and Innovation Laboratory (MoTILa), Monash University, Clayton 3168, Victoria, Australia

Corresponding author: Natalie LY Ngu, MBBS, Doctor, Department of Gastroenterology and Hepatology, Monash Health, Level 3, 246 Clayton Road, Clayton 3168, Victoria, Australia. natalie.ngu@monash.edu

Abstract

Acute-on-chronic liver failure (ACLF) is a poorly defined syndrome characterised by rapid clinical deterioration in patients with chronic liver disease. Consequences include high short-term morbidity, mortality, and healthcare resource utilisation. ACLF encompasses a dysregulated, systemic inflammatory response, which can precipitate extra hepatic organ failures. Common precipitants include infection, alcoholic hepatitis, and reactivation of viral hepatitis although frequently no cause is identified. Heterogenous definitions, diagnostic criteria, and treatment guidelines, have been proposed by international hepatology societies. This can result in delayed or missed diagnoses of ACLF, significant variability in clinical management, and under-estimation of disease burden. Liver transplantation may be considered but the mainstay of treatment is organ support, often in the intensive care unit. This review will provide clarity around where are the controversies and consensus in ACLF including: Epidemiology and resource utilisation, key clinical and diagnostic features, strategies for management, and research gaps.

Key Words: Acute-on-chronic liver failure; Liver cirrhosis; End stage liver disease; Epidemiology; Mortality

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Core Tip: Acute-on-chronic liver failure is characterised by rapid clinical deterioration in patients with chronic liver disease. Consequences include high short-term morbidity, mortality, and healthcare resource use. Heterogenous definitions, diagnostic criteria, and treatment guidelines create further challenges to optimal care. This review summarises epidemiology and resource utilisation, key clinical features, strategies for management, and research gaps.

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INTRODUCTION

Acute-on-chronic liver failure (ACLF) is a well-recognised syndrome of rapid clinical deterioration in those with chronic liver disease (CLD). It is associated with high short-term mortality of 22%-74%[1-3]. The prevalence and impact of ACLF is likely underestimated as there is no consensus definition nor diagnostic criteria[4]. The systemic inflammatory response in ACLF often results in extra-hepatic organ failures and frequently necessitates intensive care unit (ICU) level support[5]. The key management principles in the acute phase of ACLF includes the diagnosis and treatment of underlying triggers such as infection, and provision of organ support[1,6,7]. For those surviving to hospital discharge, there is limited guidance for management in the post-admission period and although comparable evidence is scarce, re-admission rates are likely to be 30%-40%[8,9]. In this article, we review the current areas of controversies and consensus with regards to ACLF epidemiology, economic impact, clinical manifestations, diagnostic criteria, and management principles.

EPIDEMIOLOGY

In European populations, the CANONIC study[1] demonstrated 30% prevalence amongst patients admitted with cirrhosis, of which ACLF was the presenting complaint in 20% of hospital admissions using the European Association for the Study of the Liver criteria. In North America, one study demonstrated a 24% prevalence using the North American Consortium for the Study of End Stage Liver Disease criteria, which includes bacterial infection as a criterion[10]. In a study of 565 patients who underwent liver transplantation in Shanghai, China; 41% had ACLF according to Asian Pacific Association for the Study of the Liver (APASL) criteria[11].

The primary aetiology of underlying CLD and the precipitant for ACLF is likewise reflective of local region and definitions. Historically, chronic hepatitis B through vertical transmission has been the most frequent cause of CLD, particularly in Asian populations from which the APASL criteria was derived (49%-59%)[12,13]. Globally, alcohol use disorder has likely overtaken chronic hepatitis B as the most common aetiology for CLD and precipitant of ACLF[13]. The most common precipitating events for ACLF in order of frequency are bacterial infections, alcohol excess, and hepatitis B reactivation[14,15]. The latter remains the most common precipitant for ACLF in Asia[12]. The absence of an identifiable precipitant in up to 40% of patients is a significant contributor to the diagnostic uncertainty and variability in criterion applied[1].

ECONOMIC IMPACT

There is limited data on the total economic burden of ACLF, although this has been explored for cirrhosis and chronic liver disease in Australia[16] and internationally[17-19]. Current cost estimations are extrapolated from cirrhosis populations and mostly reported from the healthcare payer/provider perspective[6]. The indirect costs of ACLF such as lost productivity and disability may be significant, but its value has not been extensively quantified[20]. Whilst hospital-based costs or direct healthcare related costs can be theoretically analysed through application of diagnostic criteria or International Classification of Disease coding to health service records, there is a paucity of data on indirect costs related to disability, impact on carers, and premature mortality.

Direct healthcare costs are related to the number of organ failures, need for ICU support, and total length of stay. Each of these direct cost components are disproportionately higher in ACLF compared to decompensated cirrhosis alone[7]. A population-based cohort study in Thailand demonstrated a 3.5-fold average cost of hospitalization for ACLF compared to hepatic decompensation, using the North

American Consortium for the Study of End-Stage Liver Disease (NACSELD) definition of ACLF of two or more extrahepatic organ failures in patients with cirrhosis[7]. A national inpatient database study from the United States similarly demonstrated increasing annual liver-related hospital expenditure between 2001 and 2011. Inpatient costs increased 2-fold for cirrhosis to $9.8 billion and 5-fold for ACLF to $1.7 billion[6]. The global trend of an increasing prevalence of CLD and incidence of ACLF worldwide[17,21] will further compound the economic burden for healthcare systems.

There are no proven cost-effective interventions for ACLF treatment, which is primarily supportive care in addition to addressing underlying aetiologies and precipitants[19]. Treatments evaluated include intravenous human albumin transfusions which have not demonstrated a mortality benefit in ACLF[22] and variable mortality benefit in hepatic decompensation[23,24]. Indirect ACLF-related costs have been captured in large studies on the global burden of CLD. These suggest a 1.5% contribution to all disability-adjusted life years in 2016, which was more pronounced in countries with a lower socio-demographic index[17,25]. This data may overlook discrepancies in outcomes in countries such as Australia with a higher socio-demographic index as a proxy of development, where Indigenous Australians with cirrhosis have disproportionately higher rates of hospital re-admission and death than non-Indigenous Australians[26]. To reduce healthcare-related costs, interventions for ACLF must achieve the Holy Grail of reduced short-term mortality, length of stay, readmission, and organ failure. Additionally, early use of prognostic scores such as NACSELD-ACLF[10] or chronic liver failure (CLIF)-C ACLF[27] should be routinely applied to accurately predict those who with a poor prognosis and may be better suited to palliative care and thus reduce ineffectual resource allocation.

CLINICAL MANIFESTATIONS AND DIAGNOSTIC CRITERIA

There is no universally accepted set of diagnostic criteria for ACLF, with variable criteria identified by four major international hepatology associations[28,29]. Lack of a consensus remains problematic with the potential for delayed or missed diagnoses, and challenges in applying evidence-based treatment. There is clinical consensus that ACLF is a distinct syndrome to acute hepatic decompensation, however, patients may initially present with clinical features of a decompensating event including worsening of abdominal ascites, jaundice, gastrointestinal bleeding and hepatic encephalopathy (HE)[30]. Features of bacterial infection, such as urinary tract infection, pneumonia, or spontaneous bacterial peritonitis, with may also be present[5]. Organ failure is a hallmark of ACLF and can include renal failure and manifestations of this (such as uremia, acidosis, oliguria), respiratory and circulatory failure[2]. Beyond these non-specific clinical manifestations, the regionally relevant set of diagnostic criteria diverge in the exact thresholds and subtypes of how and what they classify as ACLF.

The *World Gastroenterological Organisation* (WGO) has proposed criteria to identify clinical, prognostic, and pathophysiologic subtypes[3] and define ACLF as an independent syndrome. Five requirements have been stipulated including: (1) Distinction from acute liver failure; (2) distinction from hepatic decompensation; (3) definition of pathophysiology; (4) definition of specific clinical signs and laboratory tests to confirm diagnosis and exclude other disease; and (5) a validated scoring system to assess severity. A system categorising ACLF into three subtypes is shown in Table 1.

The APASL criteria includes a serum bilirubin level ≥ 50 mg/L and International Normalized Ratio (INR) ≥ 1.5 complicated by ascites and/or encephalopathy within 4 wk in a patient with previously diagnosed or undiagnosed chronic liver disease or cirrhosis[31].

The European Association for the Study of the Liver (EASL) and the CLIF consortium definition requires concomitant organ failure and provides prognostication guidance according to the grading of severity[1,27]. ACLF is explicitly excluded in the absence of extra-hepatic organ failure, defined as renal failure with serum creatinine ≥ 2.0 mg/dL or single non-kidney organ failure with HE to meet criteria for low grade ACLF[1]. ACLF severity grading and criteria are summarised in Table 2, with the scoring system and organ system involvement shown in Table 3. The 28-d mortality is graduated from 23.3% in grade 1 to 75.5% in grade 3[1]. Most patients meeting ACLF criteria in the latter cohort required intensive care unit support, highlighting the greater disease severity and associated resource utilisation in this diagnostic system.

The NACSELD criteria was developed as a bedside tool to predict 30-d survival in hospitalised patients with cirrhosis with decompensation in the context of infection[11,32]. The NACSELD-ACLF is defined as two or more of the following organ failures: Brain failure (West-Haven grade 3 or 4 encephalopathy), renal failure (need for renal replacement therapy), respiratory failure (need for bilevel positive airway pressure or mechanical ventilation), and shock (the need for vasopressor support, mean arterial pressure < 60 mmHg, or a reduction of > 40 mmHg in systolic blood pressure from baseline despite adequate fluid resuscitation). Validation studies have demonstrated that the NACSELD-ACLF predicts survival in infected and uninfected hospitalised patients with cirrhosis, and similarly to EASL criteria, demonstrates that the number of organ failures strongly predicts survival[10].

The WGO clinical sub-types were proposed early in the identification of ACLF as a distinct clinical entity and are a useful bedside tool. However, WGO criteria have limited correlation with prognosticating mortality and resource use[3]. The other three definitions (APASL, CLIF-C ACLF and

Table 1 World Gastroenterological Organisation definitions of acute-on-chronic liver failure subtypes[30]

Type A-noncirrhotic	Type B-compensated cirrhosis	Type C-decompensated cirrhosis
Acute flare of noncirrhotic CLD resulting in liver failure including hepatic encephalopathy	Rapid deterioration of previously well-compensated cirrhosis after major insult such as hepatitis (drug, viral, alcoholic), infection, or surgery	Rapid deterioration in those with previous hepatic decompensation

CLD: Chronic liver disease.

Table 2 The European Association for the Study of the Liver and chronic Liver Failure Consortium grading of acute-on-chronic liver failure severity[12,25]

ACLF Grade	Criteria
No ACLF	No organ failure or; one organ failure (liver, coagulation, circulatory, respiratory) with serum creatinine < 1.5 mg/dL and no HE or single cerebral failure and serum creatinine < 1.5 mg/dL
Grade 1	Single kidney failure or single liver, coagulation, circulatory, or respiratory failure + serum creatinine 1.5-1.9 mg/dL and/or HE I-II or single cerebral failure (HE III-IV) + serum creatinine 1.5-1.9 mg/dL
Grade 2	2 organ failures
Grade 3	3 or more organ failures

HE: Hepatic encephalopathy; ACLF: Acute-on-chronic liver failure.

Table 3 Defining organ/system failure using Chronic Liver Failure-Acute-on-Chronic Liver Failure Sequential Organ Failure Assessment scoring[12]

Organ system	Parameter	Score = 1	Score = 2	Score = 3
Liver	Serum bilirubin (mg/dL)	< 6	6-12	> 12
Kidney	Serum creatinine (mg/dL)	< 2	2.0-3.5	≥ 3.5 or renal replacement therapy
Brain	West-Haven grade	0	I-II	III-IV
Coagulation	INR	< 2.0	2.0-2.5	≥ 2.5
Circulation	MAP (mmHg)	≥ 70	< 70	Vasopressors
Respiratory	PaO2/FiO2	> 300	≤ 300 and > 200	≤ 200
	OR SpO2/FiO2	> 357	> 214 and ≤ 357	≤ 214

INR: International Normalized Ratio; MAP: Mean arterial pressure, mmHg millimeters of mercury; PaO2: Partial pressure of arterial oxygen; FiO2: Fraction of inspired oxygen; SpO2: Pulse oximetric saturation.

NACSELD) have better correlation with mortality, primarily due to correlation with objective biochemical parameters, and organ failures with respect to the CLIF-C ACLF criteria. The APASL criteria require the presence of CLD but not necessarily cirrhosis, and that the acute precipitating event must be liver-related[33]. Conversely, CLIF-C ACLF criteria stipulates the presence of underlying cirrhosis, extra-hepatic organ failures and the acute precipitating event can be of non-hepatic origin[32]. Therefore, ACLF populations identified using APASL criteria may include more patients with hepatic decompensation, who may not have the same short-term mortality and economic burden as those with ACLF defined otherwise by EASL. The NACSELD criteria incorporates organ failures but does not specify values for pulse oximetry or arterial blood gases to guide ventilation and therefore is potentially more vulnerable to subjectivity compared to EASL criteria. Recent clinical guidelines published by Bajaj *et al*[34] suggest that none of the current sets of criteria are adequate to inform management. In summary, organ failure appears to be an important marker of mortality in ACLF and is a component of diagnostic criteria for two of the four major definitions described. Standardisation of ACLF definition and management protocols is a critical unmet clinical need. It is the cornerstone to prompt diagnosis, evidence-based management, and reduced population heterogeneity in the research setting.

MANAGEMENT PRINCIPLES

Despite high short-term mortality[35,36], ACLF management is primarily supportive and focuses on reversing organ failure. The key pathophysiologic drivers of systemic inflammation and paradoxical immunoparesis have no specific therapy at present[36], so addressing the precipitating factors, prevention and management of end-organ complications, and targeted organ support constitute the foundations of care[4,31]. However, more than 30% of patients still progress to multiorgan failure and death within 30 d of diagnosis. Model for End-Stage Liver Disease including serum sodium (MELD-Na) and Child-Pugh Scoring systems have limited prognostic integrity as they do not account for the cerebral, respiratory or circulatory dysfunction that accompanies ACLF[5,31].

The APASL ACLF Research Consortium (AARC) ACLF score is a prognostic model constructed from the AARC database. It incorporates five variables, including lactate, grade of HE, INR, bilirubin and serum creatinine levels[37] to stratify patients into grade 1 (score of 5-7), grade 2 (score of 8-10) or grade 3 ACLF (score of 11-15), with 28 day mortality rates of 12.7%, 44.5%, and 85.9% respectively[30]. Grade I ACLF are said to have potential recovery, grade II require intensive monitoring and grade III need immediate intervention and consideration of transplantation[30]. Scores of > 10 should be considered for transplant. This has been predominantly validated in an Asian population. Alternate scoring systems include the CLIF-ACLF SOFA score and the CLIF-Consortium ACLF scores (CLIF-C ACLF). These dynamic scoring systems allow for better prognostication of 28-d mortality rate, hence assisting stratification of ACLF[38-40]. The CLIF-C ACLF score incorporates the CLIF-C Organ Failure score (bilirubin, creatinine, INR, West-Haven grade for encephalopathy, mean arterial pressure and PiO2/FiO2 ratio), along with age and white cell count[5,41]. This has been validated as a prognostic tool in ACLF[2,40], with emerging evidence for its use in guiding treatment options. A CLIF-C ACLF score > 70 represents a subgroup in whom defining limitations of care and futility is important given their very poor predicted outcomes[30,39,41].

Increasingly there appears to be a role for liver transplantation (LT) in a select cohort of these critically ill patients. The percentage of LT performed for ACLF varies significantly between transplant centres and even within regions[4,41,42], which reflects the considerable debate around the concept of liver transplant as a therapeutic strategy. The median transplant-free mortality rate in ACLF is 30%-40% at 28-d[1,40], increasing to 75% for grade 3 ACLF[1], and 40%-60% overall at 6 mo[40,42]. A recent consensus[43,44] developed by 35 international experts from North America and Europe suggested that contraindications to transplant include PaO2/FiO2 < 150 mmHg, noradrenaline dose > 1 μg/kg/min and/or serum lactate > 9[43]. Those who recover from their initial ACLF event are at high risk of recurrent and more severe ACLF in the future[45]. Whilst at this stage transplantation in advanced ACLF is the only curative intervention available, it is associated with higher postoperative complications and longer ICU and hospital stays compared to other indications[30]. Whilst scoring systems are useful in defining timing for LT escalation and features suggesting futility, clinical and ethical challenges remain in the referral and activation of appropriate candidates.

Common precipitating events in ACLF include bacterial infection, alcoholic hepatitis, gastrointestinal bleeding, HE, and reactivation of hepatitis B in endemic regions[41,46]. In European cohorts, bacterial infection is the most common precipitant[30,41], including spontaneous bacterial peritonitis, urinary tract infections and pneumonia[30]. Bacterial infection also predicts the development of organ failure in ACLF[31], hence early detection and treatment of infection are imperative. ACLF patients have higher rates of multi-drug resistant bacteria and demonstrate a lower infection resolution rate[12]. Antimicrobial choice should incorporate local guidelines and involve prompt initiation of empiric broad spectrum antibiotics whilst awaiting sensitivity profiles[4,31,44].

Acute Kidney Injury (AKI) is a frequent feature of ACLF and considered a strong predictor of poor survival in the short and long term[36,47,48]. There is significant overlap between hepatorenal syndrome AKI (HRS AKI) and non-HRS AKI in ACLF. Isolated HRS is believed to only represent a fraction of ACLF renal complications[38]. The management of renal dysfunction in ACLF requires the exclusion of reversible causes, including nephrotoxic contributors, and optimising circulating blood volume to ensure adequate renal perfusion[38,49]. Volume expansion with intravenous albumin and continuous intravenous terlipressin is recommended for those meeting HRS-AKI criteria[49]. Continuous terlipressin infusion is preferable to bolus regimes due to the improved tolerability and reduction in adverse effects[38,49]. Noradrenaline is a possible alternative to terlipressin, with a 2016 meta-analysis of four studies (154 patients) demonstrating no superiority with regards to survival in patients treated with terlipressin *vs* noradrenaline[50]. Renal replacement therapy has historically been restricted to patients with AKI who fail the above methods and have clinical or laboratory indications as per the general AKI guidelines, and this has been translated to the ACLF population given the lack of validated data around this specific cohort[38,49]. There are also unanswered questions regarding the specific benefit of rapid correction of electrolyte abnormalities and hyperammonemia in the ACLF cohort[38].

HE in ACLF is associated with higher mortality, correlating with increasing grades of HE[51]. Management involves identification and treatment of precipitants as well as specific measures for reducing hyperammonaemia and systemic inflammation[31]. Treatment of concomitant infection, drugs and electrolyte abnormalities must always be considered and excluded[38]. Cerebral imaging should be

performed to exclude an alternative cause of altered neurology, especially given the increased risk of bleeding and clotting in this cohort[38]. Ammonia lowering therapies are the cornerstone for managing HE with lactulose as the first line agent (oral, nasogastric or rectal preparations) followed by Rifaximin as second-line therapy[38]. Continuous veno-venous haemofiltration use in acute liver failure has been associated with clinically significant reductions in serum ammonia levels and is a recognised treatment for HE in these patients[52]. There have been no large randomised controlled trials to elucidate the role for haemofiltration or haemodialysis in lowering serum ammonia levels in ACLF[38].

Variceal and other types of bleeding can precipitate ACLF and should be managed similarly to those with decompensated CLD. Non-selective beta-blockers should be continued even in patients with ascites[30,31,53]. Their use is thought to reduce systemic inflammation and have favourable effects beyond their potential haemodynamic benefits[31,53] and should only be ceased in those with haemodynamic instability[44]. Circulatory failure should be managed with volume expansion, and if haemodynamic instability persists, the use of vasopressors, aiming for a mean arterial pressure of ≥ 65 mmHg[44]. Bleeding in the ACLF cohort is predominantly secondary to portal hypertension whilst spontaneous haemorrhage is rare[54]. Historic plasma-based coagulation tests are poor predictors of bleeding in chronic liver disease[55,56]. Newer viscoelastic assays, such as thromboelastography and rotational thromboelastometry assess whole blood, which may be superior and preferential to standard laboratory testing in clinical practice but their role in ACLF management is poorly defined[38,57].

CONCLUSION

ACLF is a distinct and severe clinical entity, separate from hepatic decompensation, with high short-term mortality, healthcare resource utilisation, and poorly defined treatment goals. Clinical diagnosis and management are limited by variable definitions and diagnostic criteria. Future focuses for research should including investigating and defining specific clinical and biomarkers for prognostication and classification of ACLF subtypes, standardisation of prognostic scores for both clinical management and population stratification in clinical trials, and further evidence to support the role for liver transplantation in a well-defined cohort most likely to demonstrate long term benefit.

FOOTNOTES

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Country/Territory of origin: Australia

ORCID number: Natalie L Y Ngu 0000-0002-9383-3903; Eliza Flanagan 0000-0002-0301-135X; Sally Bell 0000-0001-8437-2124; Suong T Le 0000-0003-3305-4999.

Corresponding Author's Membership in Professional Societies: Gastroenterological Society of Australia.

S-Editor: Chen YL
L-Editor: A
P-Editor: Chen YL

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REVIEW

Liver injury in COVID-19: Clinical features, potential mechanisms, risk factors and clinical treatments

Shu-Wu Zhao, Yi-Ming Li, Yi-Lin Li, Chen Su

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Shu-Wu Zhao, Department of Anesthesiology, Hunan Cancer Hospital/The Affiliated Cancer Hospital of Xiangya School of Medicine, Changsha 410013, Hunan Province, China

Yi-Ming Li, School of Basic Medical Science, Naval Medical University/Second Military University, Shanghai 200433, China

Yi-Lin Li, Department of Pathology, Hunan Cancer Hospital/The Affiliated Cancer Hospital of Xiangya School of Medicine, Changsha 410013, Hunan Province, China

Chen Su, Department of Anesthesiology and Pain, Hunan Cancer Hospital/The Affiliated Cancer Hospital of Xiangya School of Medicine, Changsha 410013, Hunan Province, China

Corresponding author: Chen Su, MD, PhD, Assistant Professor, Doctor, Department of Anesthesiology and Pain, Hunan Cancer Hospital/The Affiliated Cancer Hospital of Xiangya School of Medicine, No. 283 Tongzipo Road, Yuelu District, Changsha 410013, Hunan Province, China. suchen@hnca.org.cn

Abstract

The coronavirus disease 2019 (COVID-19) pandemic has been a serious threat to global health for nearly 3 years. In addition to pulmonary complications, liver injury is not uncommon in patients with novel COVID-19. Although the prevalence of liver injury varies widely among COVID-19 patients, its incidence is significantly increased in severe cases. Hence, there is an urgent need to understand liver injury caused by COVID-19. Clinical features of liver injury include detectable liver function abnormalities and liver imaging changes. Liver function tests, computed tomography scans, and ultrasound can help evaluate liver injury. Risk factors for liver injury in patients with COVID-19 include male sex, preexisting liver disease including liver transplantation and chronic liver disease, diabetes, obesity, and hypertension. To date, the mechanism of COVID-19-related liver injury is not fully understood. Its pathophysiological basis can generally be explained by systemic inflammatory response, hypoxic damage, ischemia-reperfusion injury, and drug side effects. In this review, we systematically summarize the existing literature on liver injury caused by COVID-19, including clinical features, underlying mechanisms, and potential risk factors. Finally, we discuss clinical management and provide recommendations for the care of patients with liver injury.

Key Words: Liver injury; COVID-19; Clinical feature; Risk factor; Treatment and

management strategy

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Core Tip: A growing body of evidence suggests that patients with coronavirus disease 2019 (COVID-19) may experience varying degrees of liver injury. The characteristics and mechanisms of liver injury associated with COVID-19 are not fully understood. In this review, we summarized the clinical features, mechanisms, and management strategies of liver injury associated with COVID-19. Moreover, we collected all the information about high risk factors for liver injury from COVID-19, which is of significance and help for further study of liver damage related to severe acute respiratory syndrome coronavirus 2.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus. Since the first outbreak in late December 2019 in China, it has unleashed a matchless public health crisis worldwide. The COVID pandemic has been going on for nearly 3 years, and there is still no end in sight. Initially, it was considered solely an atypical pneumonia until patients started to show signs of multiorgan involvement[1]. Now we know that the effects of COVID-19 on the body are extensive. In addition to the respiratory system, almost all systems in the body, including the circulatory system, cardiovascular system, urinary system, gastrointestinal and hepatobiliary system, endocrine system, nervous system, ophthalmic system, and skin system can be affected[2,3]. SARS-CoV-2 virus mainly affects the respiratory system, causing common symptoms such as fever, fatigue, cough, and dyspnea. Relatively, diarrhea, myalgia, hemoptysis, and sore throat are less common[4]. Other reports show that liver dysfunction is a common manifestation of COVID-19 and is associated with higher mortality[5]. It is worth mentioning that the incidence of liver injury in severe COVID-19 cases can reach 93%[6]. However, the exact mechanism of how COVID-19 impairs liver function remains unclear. This comprehensive literature review is aimed at providing useful guidance for diagnosis, risk factor identification, and management of liver injury associated with COVID-19.

CLINICAL FEATURES OF LIVER INJURY IN COVID-19

Liver injury is mainly manifested as abnormal liver function (ALF) indexes. Alterations in hepatocyte damage biomarkers (HDBs), such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT), are commonly used to evaluate COVID-19-related liver injury[6,7]. In some cases, elevated lactate dehydrogenase (LDH), hypoproteinemia, prolonged prothrombin time, total bilirubin (TBil), and direct bilirubin (DBiL) are also used to assess liver function in COVID-19 patients[8-10].

In COVID-19 patients, transaminase elevations are usually mild [1-2 times the upper limit of normal (ULN)][9]. These changes in laboratory values may persist for a long time, even after hospital discharge. ALF was defined as at least one test HDB exceeding the ULN. Xu *et al*[10] evaluated the proportion of patients with abnormal HDBs, and found on admission ALT 13.2%, AST 8.5%, ALP 2.0%, GGT 7.4%, LDH 37.6%, TBiL 4.0%, DBiL 7.8%, and albumin 10.1%, and peak during the hospitalization ALT 29.4%, AST 17.5%, ALP 2.6%, GGT 13.4%, LDH 49.4%, TBiL 10.1%, DBiL 18.0%, and albumin 30.6%. In another study, the proportion of patients with at least one of the HDBs and TBil exceeding the ULN for the first time immediately after hospitalization, before discharge, a median of 14.0 d after discharge, and 1 year after discharge was 32.2%, 45.8%, 54.8%, and 28.8%, respectively[11]. In addition, a single-center prospective cohort study found that the proportion of patients with any ALF was 25.1% at 1 mo, 13.2% at 3 mo, 16.7% at 6 mo, and 13.2% at 12 mo after discharge[12]. Based on these data, long-term monitoring of liver enzymes may be warranted in patients with a history of COVID-19.

AST is generally considered to be less specific for liver injury than ALT due to additional extra-hepatic production[13,14]. Nevertheless, in liver damage, elevated AST levels appear earlier, and the increase in AST levels at admission is usually more pronounced than ALT levels. In cases of severe

COVID-19, however, ALT levels typically rise rapidly, exceed the ULN value and peak within 10-15 d of admission. Subsequently, ALT levels remained stable in all patients with liver injury and then gradually decreased with longer hospital stay. ALT is a more effective indicator of liver injury in COVID-19 patients with severe manifestations[15]. However, if serum AST and LDH levels are elevated but ALT levels remain normal, other causes of elevated liver biochemical responses rather than liver injury should be considered, such as myositis (especially AST > ALT), cardiac injury, ischemia, and cytokine release syndrome (CRS)[16].

The reported prevalence of liver injury in COVID-19 patients varied widely across studies, ranging from 4.8% to 78%[17]. This is mainly due to a variety of factors, including dynamic changes in liver function, small sample sizes, different admission criteria, lack of adjustment for baseline chronic liver disease (CLD), use of different HDBs, and inconsistent definitions of "liver injury"[10,18-20]. Notably, almost all studies were conducted on hospitalized patients, ignoring non-hospitalized patients, thus resulting in unclear overall morbidity (Table 1).

LIVER INJURY AND PROGNOSIS

COVID-19 patients with moderate or severe liver injury (SLI) have an increased risk of admission to the intensive care unit (ICU), disease progression, and death compared with patients without elevated liver chemistries[9,19,23]. Cai *et al*[6] have reported that patients with liver injury have a 9-fold greater risk for developing severe COVID-19. In a retrospective cohort study, when compared with moderate liver injury (2-5 ULN) and no/mild liver injury (< 2 ULN), COVID-19 patients with SLI (ALT > 5 ULN) had more severe clinical outcomes, including higher ICU admission rates (69% *vs* 42% *vs* 16%), intubation (65% *vs* 38% *vs* 13%), renal replacement therapy (33% *vs* 15% *vs* 7.5%), and mortality (42% *vs* 23% *vs* 21%). Among SLI patients, 70% required vasopressors, 12% received inotropes, 39% were paralyzed, 10% were proned, and 2.8% required extracorporeal membrane oxygenation[19].

Changes in liver function are predictors of severity and mortality in patients with COVID-19[5,23]. Abnormal liver biochemical parameters are closely related to an increased risk of mortality in critically ill patients with COVID-19. The levels of ALT, AST, GGT, LDH, TBil, and DBil in severe patients were significantly higher than those in mild-moderate patients. Conversely, severe patients had significantly lower albumin levels than non-severe patients[5,20]. In a study of 151 hospitalized patients, 5 liver injury parameters, ALT, AST, TBil, DBil, and indirect bilirubin, were identified as notable prognostic factors, while total protein, albumin, ALP, GGT, and total bile acid appeared to be less related to prognosis[25]. In other studies, low albumin is also a marker of severe infection and poor prognosis[10,26]. Lei *et al*[15] emphasized the association of ALF tests, especially AST and TBil, with higher mortality. They observed that AST was more frequently elevated than ALT in severe patients. However, elevated ALP and peak ALT were significantly associated with discharge to hospice and death[19,27].

ABDOMINAL IMAGING FINDINGS

Possible imaging signs of liver damage on computed tomography (CT) scans of the hepatobiliary system include hepatomegaly, decreased liver density, periportal edema, fat stranding around the gallbladder, portal lymphadenopathy, and dilated gallbladder and bile ducts[28,29]. Portal venous gas can be seen in patients with mesenteric ischemia, especially in critically ill patients[30]. CT-quantified liver density can be assessed by the liver-spleen attenuation ratio, which correlates with the severity of liver injury. A common manifestation of liver damage caused by COVID-19 is homogeneous or heterogeneous low density of the liver. Liver hypodensity is more common in critically ill cases[28]. Ultrasound can be easily performed in COVID-19 patients to help identify liver damage quickly and effectively. The most frequent sonographic finding is hepatomegaly with increased parenchymal echogenicity, followed by biliary disease, including gallbladder sludge and distention, gallbladder wall thickening, mural hyperemia, intraluminal mud, and pericholecystic fluid[29-31]. Portal venous gas suggests mesenteric ischemia. Further, gallbladder cholestasis is common in critically ill patients of COVID-19[30]. Collectively, imaging of liver injury can reveal changes in liver density, gallbladder and bile duct dilation, portal pneumatosis and/or mesenteric ischemia.

PROPOSED MECHANISMS OF LIVER INJURY

The pathological basis of liver injury following COVID-19 infection is puzzling and not fully understood. Studies suggest that direct cytotoxicity, hypoxic hepatitis, cytokine storm syndrome, exacerbation of preexisting liver disease, and drug-induced liver injury (DILI) may be major mechanisms of COVID-19-related liver injury.

Table 1 Criteria, grading, and incidence of abnormal liver function or injury

Ref.	Sample size	Study type	Criteria and grading of ALF or injury	Comments
Salık *et al*[5]	533	Retrospective study	Liver biochemical parameters: ALT, AST, and TBiL > ULN. Liver injury: ALT and/or AST > 3 ULN, and/or TBiL > 3 ULN	NA
Cai *et al*[6]	417	Retrospective, single-center study	ALF: > ULN. Liver injury: ALT and/or AST > 3 ULN, ALP, GGT, and/or TBiL > 2 ULN	76.3% had ALF and 21.5% had liver injury during hospitalization
Fan *et al*[8]	148	Retrospective, single-center study	Increased levels of ALT, AST, GGT, ALP, and total bilirubin	37.2% had ALF at hospital admission
Kulkarni *et al*[9]	20874	Meta-analysis	ELC: AST or ALT > ULN. SLI: Any elevation of enzymes > ULN and bilirubin over 2 ULN	ELC: 23.1% at initial presentation. 24.4% developed ELC during the illness
Xu *et al*[10]	1003	Retrospective cohort study	Mild liver injury: 1-2 ULN. Moderate liver injury: 2-5 ULN. Significant liver injury: > 5 ULN	Most patients with abnormal liver function parameters had mild elevations (1-2 ULN) at admission and peak hospitalization
Hundt *et al*[13]	1827	Retrospective observational cohort study	ELC: AST, ALT, ALP, TBiL, albumin: > ULN	ELC at pre-hospitalization (AST 25.9%, ALT 38.0%, ALP 56.8%, and TBiL 44.4%). Admission (AST 66.9%, ALT 41.6%, ALP 13.5%, and TBiL 4.3%). Peak hospitalization (AST 83.4%, ALT 61.6%, ALP 22.7%, and TBiL 16.1%)
Balderramo *et al*[14]	298	Multicenter study	ALEx2: The elevation of at least one of the following: TBil, ALT, AST, GGT, or ALP > 2 ULN	During admission, 29.2% out of 298 patients presented ALEx2
Phipps *et al*[19]	6913	Retrospective cohort study	Mild: ALT 1-2 ULN. Moderate: ALT between 2-5 ULN. Severe: ALT > 5 ULN	Among patients who tested positive, 45% had mild, 21% moderate, and 6.4% SLI
Wang *et al*[21]	156	Retrospective, 2-centers study	Elevated aminotransferases	41.0% patients with elevated aminotransferases
Liu *et al*[22]	245	Retrospective, single-center study	Mild liver dysfunction: AST ≥ ULN. Moderate liver dysfunction: AST ≥ ULN combined with any parameter being greater than the ULN values of ALT, GGT, and TBiL. Severe liver dysfunction: AST ≥ ULN combined with ALT ≥ 3 ULN and/or GGT, TBiL ≥ 2 ULN	43.7% experienced mild liver dysfunction, 40.4% experienced moderate liver dysfunction, and 20.4% experienced severe liver dysfunction
Chaibi *et al*[23]	281	Retrospective cohort study	ALF: AST, ALT, GGT, ALP or TBil > ULN	36.3 % had liver dysfunctions. Only a minority of patients (6.4%) had perturbations above 5 times the ULN
Shousha *et al*[24]	547	Multicenter cohort study	Liver injury: Transaminase > 3 ULN	26% and 32% of patients had elevated ALT and AST, respectively. 4.91 and 3.70%patients, respectively, had AST or ALT elevation > 3 ULN

ALEx2: Abnormal liver enzymes over twice the upper limit of normal; ALF: Abnormal liver function; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ELC: Elevated liver chemistries; DBiL: Direct bilirubin; GGT: Gamma-glutamyl transferase; NA: Not available; TBil: Total bilirubin; ULN: Upper limit of normal.

Direct cytotoxicity

The dual blood supply to the liver may be a route of infection. It is speculated that retrograde liver infection occurs after intestinal infection with SARS-CoV-2[32,33]. It is known that the S protein of SARS-CoV-2 binds to the angiotensin-converting enzyme 2 (ACE2) receptor to facilitate virus entry into host cells. ACE2 receptors are widely expressed in multiple organs, including the liver[34]. Although the expression of ACE2 is much lower in hepatocytes compared to type 2 pneumocytes, its expression levels are similar in cholangiocytes and type 2 pneumocytes[35], indicating that the hepatobiliary system is a potential target organ of SARS-CoV-2.

SARS-CoV-2 RNA has been reported to be detectable in the liver of COVID-19 patients. Electron microscopy also revealed larger numbers of coronavirus particles in the livers of these patients[21,36]. Postmortem liver biopsies showed typical coronavirus particles in the cytoplasm and typical viral infection lesions, such as mitochondrial swelling, endoplasmic reticulum dilation, and decreased glycogen granules. Besides, massive hepatocyte apoptosis and some binuclear hepatocytes were also observed[21].

Cytokine storm syndrome

Cytokine storm refers to the rapid and massive production of various cytokines in body fluids, which plays an important role in acute respiratory distress syndrome and multiple organ failure. The liver

cannot escape the cytokine storm. The pathogenesis of cytokine-mediated liver injury may stem from inflammation, altered coagulation, and activation of the renin-angiotensin-aldosterone system, culminating in microvascular insult, hepatocyte damage, and perpetuation of inflammation[37]. It has been reported that plasma levels of interleukin (IL)-2, IL-6, IL-7, IL-10, interferon (IFN)-γ, granulocyte colony-stimulating factor, IFN-inducible protein-10, monocyte chemoattractant protein-1, recombinant macrophage inflammatory protein 1 alpha, and tumor necrosis factor alpha (TNF-α) were higher in severe COVID-19 patients than in mild and moderate cases[38,39].

The IL-6 signaling complex causes deleterious changes in hepatic sinusoidal endothelial cells and may promote blood clotting. This may be a possible mechanism behind liver injury in these patients [40]. Animal experiments have demonstrated that TNF-α has a moderate contribution to ALT elevation, necroinflammation, and apoptosis[41]. The role of other cytokines in liver injury in COVID-19 patients still requires further study.

Hypoxia, endotheliitis, and coagulation dysfunction

Patients with COVID-19, especially with severe manifestations, may have varying degrees of hypoxemia. Interestingly, some of them have no experience with breathing difficulties[42]. *In vivo* and *in vitro* studies have observed the occurrence of hepatic ischemia and hypoxia, hepatic cell death, and inflammatory cell infiltration[43]. Moreover, studies have found that SARS-CoV-2 enters endothelial cells, destroys vascular endothelium, and causes diffuse endothelial inflammation that can rapidly induce vasoconstriction and procoagulant tendency[44,45].

Spiezia *et al*[46] found that COVID-19 patients with acute respiratory failure presented with severe hypercoagulability rather than consumptive coagulopathy. In these patients, plasma levels of fibrinogen and D-dimer were significantly elevated and a marked hypercoagulable thromboelastometry profile was observed. Rampotas and Pavord[47] examined 20 random blood films from COVID-19 patients receiving invasive ventilation and observed the presence of platelet aggregates and macrothrombocytes, indicating increased platelet activity.

Reactivation of pre-existing liver disease

Liu *et al*[48] evaluated hepatitis B virus (HBV)-DNA viral load in 19 hospitalized patients with COVID-19. They found that three patients had HBV reactivation (HBVr) and one patient had a high HBV-DNA viral load throughout the hospital stay. This study suggests that COVID-19 patients with pre-existing chronic HBV infection, with or without corticosteroids use, may be at risk for hepatitis B reactivation. In a review, Perrillo *et al*[49] divided the drugs that induce HBVr into three categories. High-risk drugs are anticipated to induce HBVr in > 10% of cases, moderate-risk drugs are anticipated to induce HBVr in 1%-10% of cases, and low-risk drugs are anticipated to induce HBVr in < 1% of cases. Moderate/high-dose corticosteroid therapy for ≥ 4 wk is a high-risk factor for HBVr. Anthracycline derivatives are moderate/high-risk drugs. Moderate-risk drugs include TNF-α and other cytokine inhibitors, integrin inhibitors, tyrosine kinase inhibitors, and ≥ 4 wk of low-dose corticosteroid therapy. Therefore, patients receiving any of these drugs for COVID-19 are at risk of inducing HBVr and its complications.

DILI

Various potentially hepatotoxic drugs such as remdesivir, lopinavir, azithromycin, hydroxychloroquine, acetaminophen, antibiotics, and corticosteroids are thought to induce liver injury[50,51]. In some cases, the extent of liver damage depends on the dose[52]. Antiviral drugs have been used against SARS-CoV-2, examples of such antivirals are remdesivir, lopinavir-ritonavir, and others. They have all been documented to be potentially hepatotoxic. Although some small-scale trials have reported ALT/AST elevations with remdesivir, most clinical trials have not shown significant hepatotoxicity in the treatment of COVID-19[53]. Lopinavir/ritonavir and remdesivir have similar hepatotoxicity profiles [54].

Dexamethasone, used for hypoxic respiratory failure in patients with COVID-19, is known to induce the elevation of liver enzymes, increase hepatic lipid peroxidation, and decrease antioxidant activity [55]. The liver-damaging effects of azithromycin and acetaminophen have been proven for many years [56,57]. Acetaminophen, an analgesic and antipyretic drug widely used for mild-to-moderate pain and fever, may cause dose-dependent hepatotoxicity[52].

RISK FACTORS FOR LIVER INJURY

Studies have shown that the incidence of liver injury in severe/critically ill patients is much higher than the incidence in moderate cases[17,58]. Apparently, male sex, older age, and higher body mass index are also associated with liver damage from COVID-19[6,17,58,59]. Besides, coexisting diseases such as hypertension, diabetes, cardiovascular disease, malignancy, and some liver diseases may all be risk factors for liver damage[60,61]. Currently, the susceptibility of children and pregnant women to liver injury is not fully understood.

Male sex

Multiple studies show that men with COVID-19 have an increased risk of liver damage[6,17,59,62]. Among younger patients, men also have higher odds of severe pneumonia, acute kidney injury, and acute liver injury than women. However, among elderly patients, there was no difference in the likelihood of poor outcomes between men and women[62].

Possible mechanisms are attributed to the activity of sex hormones and X-linked genes and differential regulation of innate and adaptive immune responses to viral infection. Compared with women, men have higher circulating levels of ACE2 and ACE2 levels in the lungs. Moreover, testes have much higher levels of ACE2 than ovaries. Additionally, men have lower expression of protective cytokines but higher levels of pro-inflammatory cytokines and chemokines[62].

Elderly

In a study of 900 patients with COVID-19, those aged 40-69 were at particularly high risk of liver injury and liver-related death. COVID-19-related deaths were more frequent in patients 40-69 years and ≥ 70 years of age with elevated AST levels. Although only a small proportion (1.7%) of patients without prior liver disease also died from liver-related causes, severe liver impairment and acute liver failure are rare but important complications of COVID-19[63]. Liver dysfunction is associated with poor prognosis in elderly patients with higher mortality due to liver cell damage[64].

Liver transplant

According to recent reports, liver transplant (LT) patients have a higher incidence of COVID-19, possibly due to long-term immunosuppression. Despite the increased risk of acquiring COVID-19, LT patients have lower mortality rates than matched general individuals[65]. In another study, the prevalence of COVID-19 in LT patients was 6.05%, twice that of the general population of the same age, possibly due to higher susceptibility to the virus[66]. Verbeek *et al*[67] suggested that organ transplantation should be avoided in patients with active infection and respiratory symptoms because of the risk of COVID-19 progression and subsequent organ failure, as well as the risk of exposure to the virus for transplant operators.

Furthermore, patients with LT are at high risk for hepatic decompensation and increased mortality, and may suffer from severe extrahepatic sequelae of COVID-19[68,69]. Due to lack of evidence that LT children are at a greater risk of contracting COVID-19, routine withdrawal of immunosuppressive drugs is not recommended for LT children or patients with autoimmune liver disease[70]. Generally, LT recipients do not appear to have an increased risk of death following COVID-19 infection compared to the matched general population[71].

CLD

The most common cause of CLD is nonalcoholic fatty liver disease (NAFLD), followed by HBV infection, alcohol-related liver disease, and hepatitis C virus infection[72]. Liver injury and pre-existing CLD are significantly associated with disease severity and mortality in COVID-19 patients[73,74]. Yang *et al*[75] found that CLD is independently associated with COVID-19 severity and mortality, especially in a male-dominated elderly population. However, some studies believe that liver injury is indeed an independent predictor of key outcomes, but CLD and HBV infection status are not significant comorbidities of COVID-19[73,74,76].

Similar to other CLDs, metabolically associated fatty liver disease (MAFLD) has been shown to have longer viral shedding, a higher risk of disease progression, a higher all-cause mortality, and higher COVID-19-related mortality than patients without MAFLD[72,77]. Compared with other causes of CLD, patients with autoimmune hepatitis have a worse prognosis for COVID-19[78,79].

In adult studies, certain populations, such as those with cirrhosis, nonalcoholic steatohepatitis, and liver cancer, have been found to have an increased risk of severe COVID-19 and a poorer prognosis[69,80-82]. In adults with COVID-19, cirrhosis is a risk factor associated with worse outcomes. A large survey of 220727 patients found that COVID-19 infection in patients with cirrhosis was associated with a 2.38-fold risk of death, while cirrhosis in CLD patients with COVID-19 was associated with a 3.31-fold risk of death[83]. These results suggest that cirrhotic patients with COVID-19 infection are associated with an increased risk of all-cause mortality. Zecher *et al*[84] concluded that there were no differences in age, sex, autoimmune liver disease, and cirrhotic status between COVID-19 and non-COVID-19 cases.

Children with CLD, including obese children with suspected or confirmed NAFLD, may be at an increased risk for COVID-19 infection and severe COVID-19[70,85]. Children with CLD may experience decompensation of end-stage liver disease during COVID-19 infection[70]. Compared with LT recipients, children with CLD, including children with end-stage liver disease, are more likely to be hospitalized and require intensive care[86]. However, in the study by Di Giorgio *et al*[87], the susceptibility of different pediatric patient groups to COVID-19 infection was similar, and underlying liver disease may not increase the risk of severe COVID-19. The inconsistency between these findings may be related to the different sample sizes collected.

Obesity

Cumulative evidence support obesity as a risk factor for severe COVID-19 and related death, directly or indirectly increasing inflammation, hypercoagulability, and mechanical obstruction[88]. Obesity has emerged as a strong independent determinant of increased risk of morbidity and mortality in patients infected with COVID-19. In addition, data suggest that visceral obesity and hyperglycemia in non-diabetic and diabetic patients may also be significant independent risk factors for severe COVID-19[89]. In another study, patients aged 40-69 had a higher prevalence of obesity (44.4%), suggesting that a certain proportion of patients with hepatic steatosis in this age group may be predisposed to COVID-19-related liver damage[63,78,90]. Furthermore, one study showed that > 50% of COVID-19 cases in patients with underlying hepatic steatosis were severe, with a mortality rate of 17%[91].

Diabetes mellitus

Diabetes mellitus, whether due to insufficient pancreatic beta cells or peripheral insulin resistance, is considered a risk factor for COVID-19 infection. Numerous studies have shown that new-onset hyperglycemia, ketoacidosis, and diabetes are frequently observed in patients with COVID-19[88,89,92,93]. Individuals with diabetes often have associated comorbidities, such as obesity, hypertension, and cardiovascular disease, as well as diabetic complications, including chronic kidney disease, vascular disease, and related immune dysfunction, all of which put them at risk for infectious complications[94]. In a study of 458 patients with COVID-19 and diabetes, those with liver injury and chronic kidney disease had significantly higher mortality rates than other complications[95]. In other words, chronic kidney disease and liver disease are the two main contributors to the rise in mortality among patients with diabetes and COVID-19.

Malignancy

Hepatocellular carcinoma (HCC) has been identified as a predictor of poor prognosis in COVID-19 patients[72,76]. HCC is often associated with cirrhosis, suggesting that decreased immunity may increase the risk of severe COVID-19, and that infection with COVID-19 can exacerbate pre-existing liver disease, complicating cancer management[96]. Furthermore, COVID-19 vaccination is recommended for LT candidates and patients with CLD or HCC as they are susceptible to severe COVID-19[68]. Overall, cancer patients are considered to be at high risk of developing severe COVID-19 due to comorbidities and immunosuppressive status, especially among those who have recently received chemotherapy or had surgery within a month[96,97].

Hypertension

In a study of 300 patients with COVID-19, 33.2% were diagnosed with hypertension at admission. These hypertensive patients displayed higher levels of Troponin T and creatinine near hospital discharge[93]. Notably, the proportion of hypertensive patients in severe COVID-19 was significantly higher, and the mortality rate of severe patients was higher[93]. In addition, high blood pressure may increase the risk of liver damage following COVID-19 infection in elderly patients without pre-existing CLD[73].

It has been reported that hypertensive patients have a higher probability of liver damage and a poorer prognosis. The underlying mechanism may be related to the activation of the renin-angiotensin system and the damage of ACE2-positive cholangiocytes and hepatocytes, which further lead to cholangiocyte and hepatocyte-associated disorders[69,81,98,99]. ACE2-stimulating drugs for high blood pressure have been hypothesized to increase the risk of fatal COVID-19. Fang *et al*[100] reported that patients using ACE2-elevating drugs for hypertension, diabetes or heart disease are at increased risk of COVID-19 infection.

Pregnancy

Pregnant woman with COVID-19 have significantly decreased blood lymphocytes, increased neutrophils, and elevated C-reactive protein and TBil levels[101]. In the study by Deng *et al*[102], the incidence of liver injury in pregnant women infected with COVID-19 was 29.7%. Despite a higher frequency of ICU admissions, in-hospital mortality was lower among pregnant patients compared with non-pregnant patients with COVID-19 viral pneumonia, at 1.1% for pregnant women and 3.5% for non-pregnant women. Pregnancy is not an independent risk factor for in-hospital mortality in COVID-19 patients[103]. In the study by Tunç *et al*[104], COVID-19-related hospitalization rates were 24.1% in the first trimester, 36% in the second trimester, and 57.3% in the third trimester; there was no significant relationship between pregnancy duration and the need for ICU admission.

However, pregnant women may have many comorbidities, including hypertension, chronic lung disease, diabetes, and obesity, compared with non-pregnant women[103]. Pregnant patients with COVID-19 and chronic complications such as hypertension and diabetes have an increased risk of developing inflammation and liver damage[101]. Pregnant women taking antiviral drugs have several options, including continuing treatment, stopping or switching to safer drugs. Patients with high pretreatment ALT or less than 1 year of treatment prior to pregnancy have a high risk of severe hepatitis flares after cessation of antiviral agents[105].

The perinatal outcomes of all reported cases were reassuring, with 98% live births, 78% full-term births without neonatal complications, and a 20% neonatal ICU admission rate. The stillbirth rate was as low as 1.7%, and the neonatal mortality rate was 0.8%. No vertical transmission was found in 98.4% of neonates[106,107].

Children

Children with COVID-19 infection often have minimal or no increase in liver enzymes[60]. COVID-19 may impair liver function, usually resulting in transient and moderate elevations in liver markers without significant impairment of hepatic synthesis. COVID-19-infected patients with elevated ALT are at risk for a more severe disease course, including longer hospital stay and ICU stay[85]. Compared with adult patients, pediatric patients have relatively lower rates of lymphopenia, higher inflammatory markers, and possible thrombocytopenia[108].

MANAGEMENT OF LIVER INJURY IN PATIENTS WITH COVID-19

Liver injury in mild cases of COVID-19 is usually transient, self-limiting, and reversible without treatment. However, some COVID-19 patients who present with liver injury may become critically ill and require medical attention[16]. The cause of liver injury should be analyzed and judged in all patients. Initial screening includes careful review of preexisting liver disease, exposure to hepatotoxins (alcohol, drugs, herbs, and chemicals), hypoxia, and circulation status (Table 2). Liver function indicators including ALT, AST, TBil, DBiL, albumin, prothrombin activity, and international normalized ratio should be closely monitored[109,110].

Prophylactic use of hepatoprotective and enzyme-lowering drugs is not recommended[109]. Theoretically, reducing viral load with antiviral therapy is the most effective way to reduce organ damage. However, there is currently a lack of clinical data to support it, and more attention is paid to antiviral drug-related liver injury. This may be one reason for the lack of particularly effective antiviral drugs until recently.

The management of liver injury from COVID-19 is largely empirical and mainly supportive. Patients with severe liver damage associated with COVID-19 should be treated with hepatoprotective, anti-inflammatory, and jaundice-reducing agents such as glycyrrhizic acid, polyene phosphatidyl choline (PPC), bicyclol, and vitamin E[111,112]. Glycyrrhizic acid can effectively inhibit the replication and cytopathic effect of coronavirus without obvious cytotoxicity to host cells[113]. Glycyrrhizin has anti-inflammatory properties that may offer protection against liver disease[109]. PPC may be a drug that enhances the hepatoprotective function through glutathione and magnesium isoglycyrrhizinate[114].

Currently, there is no specific treatment for critically ill patients with COVID-19. Effective suppression of the host's uncontrolled immune response during cytokine storm may be a critical step in preventing disease progression and reducing mortality[115,116]. Anakinra is an IL-1 receptor antagonist that blocks the release of IL-β. A study concluded that early anakinra treatment is associated with significantly lower ICU admissions and mortality in patients with moderate/severe COVID-19[117]. Successful anakinra therapy includes treatment duration ≥ 10 d, dose ≥ 100 mg, intravenous administration, and early initiation of therapy[118]. Canakinumab is a human monoclonal anti-IL-1β specific antibody. Studies have shown that canakinumab therapy provides rapid and durable improvement in oxygenation levels, reduced proinflammatory markers and reduced need for mechanical ventilation resulting in better outcomes[119,120].

IL-6 is one of the key mediators of cytokine storm-induced damage[121]. Currently, there are two main types of IL-6 inhibitors that target IL-6 itself (siltuximab) or its receptors (tocilizumab and sarilumab)[115]. IL-6 levels drop after administration of siltuximab, suggesting that the inhibitor may reduce CRS and mortality[122]. The literature supports the early use of tocilizumab as it has been observed to lower mortality in adults with COVID-19 pneumonia[123,124] and achieve better clinical recovery at day 28[125]. In another study, clinical improvement and mortality were not statistically different between tocilizumab and standard treatment[125]. The reason may be a higher risk of bacterial or fungal infection in patients within tocilizumab application[123,124,126]. Sarilumab is a high-affinity anti-IL-6 receptor antibody. In a phase II, open-label, randomized, controlled clinical trial of hospitalized patients with COVID-19, early use of sarilumab was safe and associated with a trend for better outcomes[127]. However, in some other studies, the efficacy of sarilumab in hospitalized patients with moderate-to-severe COVID-19 has not been established[128-130]. Inhibition of IL-6-mediated signaling may not be sufficient to reduce CRS, and the answer may lie in combination therapy and interfere with other related pathways. So far, conflicting results hinder efforts to use IL inhibitors to combat COVID-19 infection[131].

Anti-TNF therapy has also shown conflicting results. In a case-cohort study, patients treated with anti-TNF-α inhibitors were hospitalized less frequently[132]. This was a systematic review and meta-analysis of COVID-19 and outcomes in patients with inflammatory bowel diseases (IBD). Compared with patients on corticosteroids, those on anti-TNF-α therapy had a lower risk of hospitalization and ICU admission. Moreover, similar results were seen in patients treated with anti-TNF-α compared to

Table 2 Treatments of liver injury in coronavirus disease 2019

Mechanisms of liver damage	Treatments	Caution	Ref.
Hepatocellular injury	Hepatoprotective, anti-inflammatory, and jaundice-reducing agents	Preventive administration is not recommended	[109, 111, 112]
Cytokine storm syndrome	Continuous renal replacement therapy. IL-1 inhibitor, IL-6 inhibitor, TNF inhibitor	IL-1 or IL-6 inhibitors could reduce inflammation; however, they have a potential to cause DILI and worsen clinical conditions	[109, 139, 140]
DILI	Prompt discontinuation or reduction of doses of suspected triggers. Medication reconciliation is important. Discontinue all non-vital therapy, redundant types/doses, modify course duration	Requires a trade-off between therapeutic effects and side effects	[109]
Reactivation of pre-existing liver disease	Continue treatment for hepatitis B and hepatitis C if already on treatment	Difficulty distinguishing between new-onset liver injury and reactivation of pre-existing liver disease	[16, 109]
Hypoxic hepatitis	Circulation and respiratory support	Higher PEEP, which may be needed to improve oxygenation, may affect cardiac output, decreasing hepatic arterial flow, thus enhancing arterial dysfunction	[139, 140]

DILI: Drug-induced liver injury; IL: Interleukin; PEEP: Positive end-expiratory pressure; TNF: Tumor necrosis factor alpha.

patients treated with mesalamine[133]. Colonic ACE2 expression was downregulated after anti-TNF-α therapy in IBD patients[134], but no liver-related data have been reported. In another meta-analysis and systematic review of 84 studies, no difference was found in the risk of hospitalization in patients receiving anti-TNF-α therapy compared to patients not receiving anti-TNF-α therapy[135]. Foods rich in vitamins, minerals, polyphenols, and other bioactive compounds may decrease inflammatory pathway activity and prevent liver damage in COVID-19 patients[136].

Corticosteroids have a dual effect. They have been associated with DILI, especially at high doses, however they are used to treat drug-induced cholestatic hepatitis and DILI associated with hypersensitivity reactions[137,138]. The only specific antidote for acute DILI remains N-acetylcysteine for acetaminophen poisoning. Glycyrrhizin, ursodeoxycholic acid, and silymarin have been used for decades to treat DILI, but success remains anecdotal[138]. The most effective treatment for suspected DILI is to discontinue drug therapy before progression to irreversible liver failure, which results in spontaneous recovery in approximately 90% of cases[139].

CONCLUSION

Nearly 3 years later, there is still no sign that the COVID-19 pandemic is over. COVID has long-term devastating effects involving multiple organs. Particular attention should be given to liver injury associated with COVID-19. There is growing evidence that liver injury is a typical long-term effect of COVID-19, especially in critically ill cases, and may require monitoring after the patient is discharged. The exact incidence and underlying mechanism of liver damage are not well known. Fortunately, most patients with mild liver damage recover without special treatment. However, SLI is believed to worsen the prognosis and increase mortality from COVID-19. Increased research efforts are needed to identify those patients at higher risk of complications, better definition of liver injury, better understanding of the pathophysiology, and effective therapies.

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Country/Territory of origin: China

ORCID number: Shu-Wu Zhao 0000-0001-7635-1702; Yi-Ming Li 0000-0001-8186-5062; Yi-Lin Li 0000-0003-0206-4713; Chen Su 0000-0002-7568-8036.

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REVIEW

COVID-19 and liver injury: An ongoing challenge

Ioanna Papagiouvanni, Serafeim-Chrysovalantis Kotoulas, Athanasia Pataka, Dionisios G Spyratos, Konstantinos Porpodis, Afroditi K Boutou, Georgios Papagiouvannis, Ioanna Grigoriou, Christos Vettas, Ioannis Goulis

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Ioanna Papagiouvanni, Fourth Department of Internal Medicine, Aristotle University of Thessaloniki, Thessaloniki 54642, Thessaloniki, Greece

Serafeim-Chrysovalantis Kotoulas, Intensive Care Unit, Hippokration General Hospital, Thessaloniki 54642, Greece

Athanasia Pataka, Department of Respiratory Medicine, G Papanikolaou Hospital, Resp Failure Unit, Aristotle University of Thessaloniki, Thessaloniki 57001, Greece

Dionisios G Spyratos, Konstantinos Porpodis, Pulmonary Department, Aristotle University of Thessaloniki, Thessaloniki 57001, Greece

Afroditi K Boutou, Pulmonary Department, G Papanikolaou Hospital, Resp Failure Unit, Aristotle University of Thessaloniki, Thessaloniki 54642, Greece

Georgios Papagiouvannis, Department of Pharmacy, School of Health Sciences, Frederick University, Nicosia 1036, Cyprus

Ioanna Grigoriou, Respiratory Failure Clinic, Papanikolaou General Hospital, Thessloniki 57001, Greece

Christos Vettas, Ioannis Goulis, Fourth Department of Internal Medicine, Hippokration General Hospital, Thessaloniki 54642, Greece

Corresponding author: Ioanna Papagiouvanni, MSc, Doctor, Fourth Department of Internal Medicine, Aristotle University of Thessaloniki, 49 Konstantinoupoleos, Thessaloniki 54642, Thessaloniki, Greece. ioanna.d.pap@gmail.com

Abstract

The new coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was identified in December 2019, in Wuhan, China. The virus was rapidly spread worldwide, causing coronavirus disease 2019 (COVID-19) pandemic. Although COVID-19 is presented, usually, with typical respiratory symptoms (*i.e.*, dyspnea, cough) and fever, extrapulmonary manifestations are also encountered. Liver injury is a common feature in patients with COVID-19 and ranges from mild and temporary elevation of liver enzymes to severe liver injury and, even, acute liver failure. The pathogenesis of liver damage is not clearly defined; multiple mechanisms contribute to liver disorder, including direct cytopathic viral effect, cytokine storm and immune-mediated hepatitis, hypoxic injury, and drug-

induced liver toxicity. Patients with underlying chronic liver disease (*i.e.*, cirrhosis, non-alcoholic fatty liver disease, alcohol-related liver disease, hepatocellular carcinoma, *etc*.) may have greater risk to develop both severe COVID-19 and further liver deterioration, and, as a consequence, certain issues should be considered during disease management. The aim of this review is to present the prevalence, clinical manifestation and pathophysiological mechanisms of liver injury in patients with SARS-CoV-2 infection. Moreover, we overview the association between chronic liver disease and SARS-CoV-2 infection and we briefly discuss the management of liver injury during COVID-19.

Key Words: COVID-19; Liver injury; Cytokine storm; Hypoxic hepatitis; Drug-induced liver injury; Chronic liver disease

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Core Tip: Liver injury is a common feature in coronavirus disease 2019 (COVID-19) patients and was associated with disease severity and prognosis. Multiple pathophysiological mechanisms are responsible for liver injury, including direct viral effect, cytokine storm, hypoxia and drug hepatotoxicity, however, further research is needed, in order, for them, to be clearly defined. Patients with underlying chronic liver disease may be more susceptible to severe acute respiratory syndrome coronavirus 2 infection; nevertheless, evidence is still limited. It is necessary to know the mechanisms of liver injury, the clinical manifestations and the effect of COVID-19 in underlying liver disease, in order to design appropriate management programs.

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INTRODUCTION

In December 2019, severe acute respiratory syndrome coronavirus 2, (SARS-CoV-2), causing respiratory infection in humans, was detected in Wuhan, China[1]. The new coronavirus was spread worldwide, resulting in coronavirus disease 2019 (COVID-19) outbreak. On March 11, 2020, the World Health Organization declared COVID-19 as a global pandemic[2]. As of September 2022, over 603 million confirmed cases and over 6.4 million deaths have been reported worldwide[3].

Most COVID-19 patients present with typical respiratory symptoms (*i.e.*, cough, dyspnea) and fever. However, abnormal liver function is often developed in patients with COVID-19, and liver injury has been related with severe disease[4,5]. Liver damage ranges from mild asymptomatic elevation of liver enzymes to severe liver injury, while a few cases of acute liver failure have also been reported[6,7].

The aim of this review is to present the prevalence and clinical manifestations of liver injury in COVID-19, to overview the potential pathophysiological mechanisms leading to liver damage and to summarize the existing literature for patients with COVID-19 and underlying chronic liver disease. Furthermore, the management of liver complications during SARS-CoV-2 infection is also briefly discussed.

PREVALENCE AND RISK FACTORS

Numerous studies have focused on liver injury induced by COVID-19 infection. However, the definition of liver injury in COVID-19 patients has not been clearly established yet. Some researchers defined it, as any increase of liver enzymes above the upper limit of normal (ULN), while others, as an increase, at least 2 or 3 times above the ULN[8-12]. Moreover, the different statistical time points across the studies, could also affect the incidence of liver injury[8]. As a consequence, the prevalence of liver damage varies across studies. Wang *et al*[13] conducted a retrospective study and found that the 41% of 156 COVID-19 patients had abnormal liver function, while, Fan *et al*[10] demonstrated that 55 out of 148 patients (37.2%) had elevated liver enzymes on admission. In a recent retrospective study of 228 patients, without chronic liver disease, 29.4% had abnormal liver function on admission; the rate increased to 56.3% during hospitalization[14]. Cai *et al*[15] defining liver injury as alanine transaminase (ALT) or

aspartate aminotransferase (AST) 3 times higher than ULN or alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total bilirubin (TBIL) 2 times higher than ULN, observed that 41% of patients had abnormal liver tests and 5% had liver injury on admission. During hospitalization, patients with abnormal liver tests and patients with liver injury increased to 76.3% and 21.5%, respectively. Ding *et al*[16], also, demonstrated the same trend of liver function, in a large retrospective cohort study of 2073 patients. On admission, 46.2% and 5.1% had abnormal liver tests and liver injury, respectively. Yet, during hospitalization, the incidence increased to 61.8% and 14.3%, respectively. Across several meta-analyses, the pooled prevalence of liver injury ranged between 19% and 27.4%[4,5,17]. Kulkarni *et al*[18], in their meta-analysis, found that the pooled incidence of abnormal liver enzymes at initial presentation, only slightly increased during the course of disease (from 23.1% to 24.4%). Wu *et al*[19] observed a similar trend; the pooled incidence of elevated liver tests on admission and during hospitalization was 27.2% and 36%, respectively.

Liver injury has been associated with severe COVID-19 disease[4,5,19-21]. Chen *et al*[22] demonstrated that patients with deranged liver function had higher risk of systemic inflammatory response syndrome (53.5% *vs* 41.3%, $P = 0.007$) and higher mortality rate (28.9% *vs* 9.0%, $P < 0.001$). In another retrospective study, elevated AST (> 3-fold ULN) was associated with higher risk of mechanical ventilation and death[23]. Moreover, Wang *et al*[24] found that the levels of aminotransferases were significantly higher in ICU patients compared to non-ICU patients (ALT: 35 *vs* 23, normal range 9-50 U/L, $P = 0.007$ and AST: 52 *vs* 29, normal range 5-21 U/L, $P < 0.001$). Kumar *et al*[4], in their meta-analysis, confirmed that liver injury was higher in patients with severe COVID-19 disease, compared to non-severe COVID-19 disease (44.63% *vs* 20.02% respectively). Furthermore, Mao *et al*[5] conducted another meta-analysis and found that patients with severe COVID-19 infection exhibited a higher risk for abnormal liver function, including increased AST and ALT. Finally, in a recent meta-analysis of 15 studies, patients with deranged liver function and/or histopathological findings of liver disease, presented a significantly higher risk of poor COVID-19 outcomes[21]. Across several studies, other risk factors for liver injury were found to be male gender, higher BMI, older age, severe lung disease and underlying chronic liver disease[11,15,25].

CLINICAL MANIFESTATIONS

In most cases, liver injury is presented as elevated liver enzymes without specific symptoms and signs. The elevation of AST, ALT and/or TBIL is a very common manifestation in COVID-19 patients, while increased GGT and/or ALP is a less usual feature, observed in later stages of the disease[6,7]. The elevation of the aminotransferases is usually mild; their level is mostly < 5 times ULN[26]. Furthermore, liver injury in COVID-19 has been noted to be transient, while hepatic biochemical tests return to normal within 2-3 wk[6]. Severe liver injury, with aminotransferases > 20 times ULN, has been observed in 0.1% of COVID-19 patients on admission and in 2% during hospitalization, while acute liver failure, induced by COVID-19, has been reported in extremely rare cases[27,28]. Febrile hepatitis, acute cholecystitis and hepatic artery thrombosis are, also, rare clinical presentations of COVID-19[29-31]. Moreover, in some cases reports, it is suggested that SARS-CoV-2 triggered a *de novo* development of immune-mediated liver disease, such as autoimmune hepatitis and primary bile cholangitis[32-35]. Interestingly, cholangiopathy, characterized by cholestasis and structural abnormalities of bile duct, has been reported in post-COVID-19 patients, who recovered from severe and critical disease[36,37].

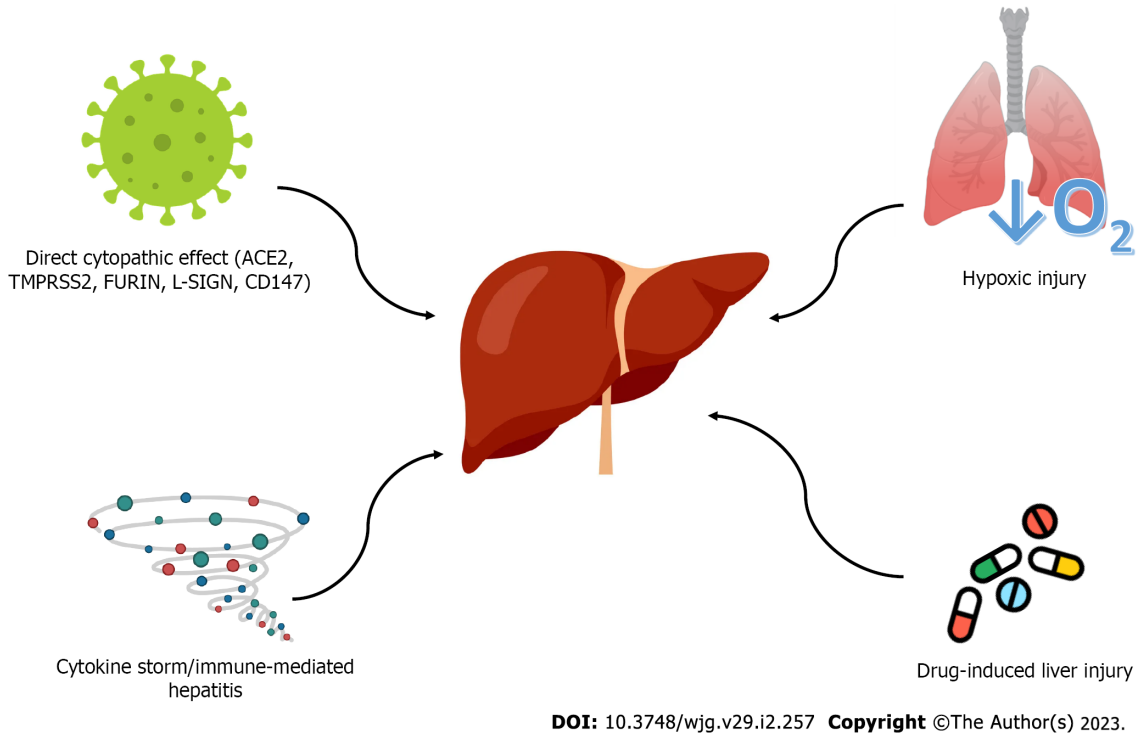
MECHANISMS OF LIVER INJURY

The pathogenesis of live injury in COVID-19 disease is still unclear. According to the available literature, the underlying mechanisms of liver injury are multifactorial and mainly, include direct viral cytopathic damage, immune-mediated hepatitis, caused by cytokine storm, hypoxia and ischemic injury and drug-induced liver toxicity. The possible pathophysiologic mechanisms of liver injury are presented in Figure 1.

Direct cytopathic effect of SARS-CoV-2

Liver is a potential target of direct SARS-CoV-2 infection. Existing literature suggests that the new coronavirus could be detected in the liver and indicates typical histological lesions related to viral infection[38]. Indeed, a series of small sample size studies demonstrated that SARS-CoV-2 RNA and viral particles are detectable in the liver of patients with COVID-19[13,39-43]. Furthermore, in a recent cohort study of 45 autopsy cases, virus RNA was detected in 69% of cases[44].

SARS-CoV-2 uses angiotensin-converting enzyme 2 (ACE2) to invade into host cells, while cell entry is facilitated by transmembrane serine protease 2 (TMPRSS2) and paired basic amino acid cleaving enzyme (FURIN)[45,46]. Single-cell RNA sequencing analysis revealed that ACE2 is expressed among different cell types in liver; in parallel, TMPRSS2 and FURIN are, also, expressed in liver cells[47-49].



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Figure 1 Mechanisms of liver injury in coronavirus disease 2019. ACE2: Angiotensin-converting enzyme 2; TMPRSS2: Transmembrane serine protease 2; FURIN: Paired basic amino acid cleaving enzyme; SIGN: Specific intercellular adhesion molecule-3-grabbing non-integrin.

The above evidence indicates that liver tissue could be susceptible to COVID-19 infection. Yet, the expression of ACE2 in bile duct cells is 20-fold higher than the expression level in hepatocytes[50]. Despite the high expression of ACE2 in cholangiocytes, which would be associated with cholestatic injury (*i.e.*, elevated levels of GGT and ALP), most studies found that hepatocellular damage is the most common pattern in COVID-19 patients (*i.e.*, elevated levels of ALT and AST)[17,51,52]. Therefore, alternative molecular pathways for liver infection cannot be excluded. The liver/Lymph node-specific intercellular adhesion molecule-3-grabbing integrin, a liver-specific capture receptor, and CD147, a receptor highly expressed in inflamed and/or pathogen-infected tissues, have been proposed as alternative receptors or enhancer factors, mediating in the SARS-CoV-2 cellular entry in the liver. Moreover, antibody-dependent enhancement may be responsible for liver infection[53]. Instead of neutralizing the virus completely, suboptimal non-neutralizing antibodies, attached to Fc receptor, promote viral entry into the liver cells[53]. In addition, existing evidence suggests that inflammatory signals [*i.e.*, interleukin-6 (IL-6), type 1 interferon] and hypoxia, related to SARS-CoV-2 infection, could result to hepatocyte regeneration, compensatory hyperplasia and upregulated expression of ACE2, leading to potentially increased hepatic susceptibility to SARS-Cov-2[45,53].

Despite that virus particles have been observed in hepatocytes and molecular pathways of virus invasion have been suggested, further evidence is needed to clearly establish the role of direct viral infection in liver injury.

Immune-mediated liver injury

COVID-19 infection can trigger uncontrolled immune response, called cytokine storm, which is characterized by exaggerated activation of immune cells and massive production of inflammatory mediators [54,55]. Indeed, pro-inflammatory cytokines [*i.e.*, IL-1β, IL-2, IL-6, IL-8, tumor necrosis factor-α, interferon-α (IFN-α), IFN-γ, granulocyte-macrophage colony-stimulating factor] were increased in severe COVID-19 disease[56]. Cytokine storm generates a process leading to tissue damage and even multiorgan failure[57]. Due to its anatomical location, liver is highly exposed to circulating cytokines, and thus, prone to inflammatory-mediated injury[58]. Furthermore, viral-induced $CD8^+$ T cells provoke the activation of Kupfer cells, resulting to T cell-mediated hepatitis[58].

Several studies have demonstrated a correlation between liver injury and increased levels of inflammatory mediators in COVID-19 patients. In a recent cohort study of 192 patients, increased IL-6 and IL-10 Levels and decreased number of $CD4^+$ T cells were identified as independent risk factors for severe liver injury[59]. Likewise, in another retrospective cohort study, inflammatory markers, such as IL-6, CRP and ferritin, were significantly higher in patients with liver injury[60]. Huang *et al*[61], conducting a retrospective study of 2623 patients, found a positive correlation between IL-6 and liver enzymes (*i.e.*, AST, ALT, and GGT), indicating that COVID-19-induced cytokine storm leads to hepatotoxicity. In

addition to that, Liao *et al*[62], suggested that, apart from IL-6, IL-2 and IL17A were also key inflammatory factors triggering liver damage.

Hypoxia-reperfusion injury

The liver is a highly aerobic organ, and, thus, it is remarkably susceptible to hypoxia[38]. Patients with COVID-19 can be complicated with respiratory failure, acute heart failure and systemic stress, causing low oxygen saturation level and/or decreased systemic arterial pressure. As a consequence, arterial perfusion and oxygenation of the liver can be reduced, leading to hepatic ischemia and hypoxia-reperfusion injury[38,63]. Furthermore, systemic inflammatory response, through microvascular dysfunction and microthrombosis, could worsen liver hypoxia[38]. Hepatic venous congestion, caused by heart failure, or high positive end-respiratory pressure, used in patients with respiratory failure, can, also, lead to hypoxic damage in the liver cells[58].

Hypoxic injury involves a biphasic process; ischemic cell damage and reperfusion-associated inflammatory response. Lipid accumulation, glycogen consumption, mitochondrial damage and increased reactive oxygen species and their peroxidation products lead to cell death, during ischemia[53]. Following ischemic injury, reperfusion induces activation of immune response and release of pro-inflammatory cytokines, resulting in further cell damage[53].

In a retrospective cohort study, hepatocellular injury pattern in COVID-19 patients was associated with hypoxia[64]. Likewise, Fu *et al*[65], in a more recent multicenter retrospective study, confirmed that patients with hypoxia were more likely to have abnormal liver function.

Drug-induced liver injury

The liver plays a crucial role in drug metabolism. Several drug metabolites induce liver cell apoptosis/necrosis and can lead to liver damage. Drug-induced liver injury (DILI) is often detected by liver enzymes tests, using the following thresholds: (1) ALT > 5 times ULN; (2) ALP > 2 times ULN; and (3) ALT > 3 times ULN and TBL > 2 times ULN[66]. Based on ALT/ALP ratio, DILI pattern can be defined as hepatocellular, cholestatic or mixed. DILI can also be intrinsic, which is dose-dependent and predictable, or idiosyncratic, which is unpredictable, with variable latency period[66]. Concerning prognosis, DILI ranges from mild to severe or even fatal, with approximately 10% of patients requiring liver transplantation[66].

At present, many drugs have been used to treat COVID-19 patients, such as corticosteroids, antiviral agents, immunoregulatory factors and antibiotics, leading to potential hepatotoxicity. Systemic corticosteroids, especially dexamethasone, were widely prescribed to both outpatients and hospitalized patients with COVID-19. Despite that, the prolonged use of corticosteroids is related to side effects (*i.e.*, infections, hyperglycemia), DILI is uncommon[67]. Corticosteroids have been associated with liver steatosis, hepatomegaly, worsening non-alcoholic fatty liver disease (NAFLD) and exacerbating HBV reactivation, however, existing literature is limited[67,68]. With regards to COVID-19, Yip *et al*[69] found that the use of corticosteroids was an independent factor of liver injury. However, this association could be explained by the fact that patients with more severe disease received corticosteroids.

Remdesivir is an inhibitor of viral RNA-dependent RNA polymerases, used in COVID-19 disease. Among its side effects, remdesivir can cause hepatotoxicity, manifested as elevated AST and ALT[67]. In most studies, 10%-50% of patients developed mild-to-moderate increase of aminotransferases, while levels > 5 times ULN were reported in 9% of patients in clinical trials[70]. Subsequently, remdesivir is contraindicated in patients with ALT > 5 times ULN or severe liver dysfunction[71]. The elevation of aminotransferases is generally reversible without clinically apparent hepatic dysfunction[67].

Tocilizumab, a humanized anti-interleukin-6 receptor (IL-6R) monoclonal antibody, is indicated in hospitalized COVID-19 patients with rapid respiratory deterioration[67]. Elevation of aminotransferases has been reported, but it is generally transient, dose-dependent, without significant liver complications [67,72]. Anakinra, an IL-1 inhibitor, has, also, been used in severe COVID-19, but hepatotoxicity is an extremely uncommon side effect. In addition to that, liver enzymes levels did not significantly differ between anakinra and placebo in clinical trials[67,73].

More recently, nirmatrelvir/ritonavir has been prescribed in COVID-19 patients, early in the course of infection, as a post-exposure protection. In clinical trials, elevation of aminotransferases was uncommon or mild in nirmatrelvir/ritonavir group and did not differ from placebo group. However, clinical data are still limited and further evidence is needed[74].

Table 1 presents the most studied drugs for COVID-19 and the existing evidence concerning their hepatotoxicity.

CHRONIC LIVER DISEASE AND COVID-19

Most studies have not provided sufficient data about the prevalence of underlying chronic liver disease (CLD) in COVID-19 patients. However, in a meta-analysis of 73 studies including 24299 COVID-19 patients, the pooled prevalence of CLD was estimated to be at 3%[75]. Patients with CLD may, already, have liver damage and SARS-Cov-2 infection is an additional "hit" to the liver, leading to further liver

Table 1 Evidence of hepatotoxicity of most studied and used drugs in coronavirus disease 2019

Drug	Mechanism of action	Characteristics of LI	Risk of DILI	DILI pattern
Corticosteroids[126]	Anti-inflammatory	Hepatomegaly, steatosis; triggering/worsening NAFLD; reactivation HBV (prolonged administration)	Low	Hepatocellular or mixed
Remdesivir[70]	Antiviral; active inhibitor of viral RNA-dependent RNA polymerases	Mild-to-moderate ALT and AST elevations; Elevation > 5 times ULN in 9% (resolved with discontinuation)	Moderate	Hepatocellular
Tocilizumab[72]	Anti-IL-6 receptor monoclonal antibody	Elevation of ALT and AST; no reports of severe LI or HBV reactivation (in COVID-19 trials)	Moderate	Hepatocellular
Anakinra[73]	IL-1 inhibitor	ALT elevation in < 1%; No association with HBV reactivation	Low	Hepatocellular
Nirmatrelvir/ritonavir [74]	Antiviral; Inhibitor of the main protease of SARS-CoV-2/protease inhibitor and potent inhibitor of the enzyme CYP 3A4	Mild ALT and AST elevation; no reports of clinical apparent LI; limited data	Low	Hepatocellular
Molnupiravir[127]	Antiviral; prodrug of the ribonucleoside analogue N-hydroxycytidine	Mild ALT and AST elevation; no reports of clinical apparent LI; limited data	Low	Hepatocellular
Low-molecular-weight heparins[128]	Anticoagulant	Mild ALT and AST elevation; LI with rapid onset and rapid recovery, without clinical symptoms	Low	Hepatocellular
NSAIDs[129]	Anti-inflammatory	Mild, transient and asymptomatic elevation of liver enzymes; more common in obese patients with comorbidities; reports of acute hepatitis (idiosyngratic, prolonged administration)	Moderate	Hepatocellular, cholestatic or mixed
Acetaminophen[130]	Analgesic and antipyretic	Dose-dependent; transient and asymptomatic elevation of ALT and AST; acute hepatitis and/or acute liver failure in overdose	High	Hepatocellular

DILI: Drug-induced liver injury; ALT: Alanine aminotransaminase; AST: Aspartate aminotransferase; HBV: Hepatitis B virus; IL-6: Interleukin-6; IL-1: Interleukin-1; LI: Liver injury; NAFLD: Non-alcoholic fatty liver disease; ULN: Upper limit of normal; NSAID: Nonsteroidal antiinflammatory drugs.

functional impairment[76]. Although patients with stable CLD, without cirrhosis, are not more susceptible to severe COVID-19, those with cirrhosis, alcoholic liver disease (ALD), hepatocellular carcinoma (HCC) and NAFLD may be in a greater risk for severe disease with liver injury and poor outcome[6,76-78].

Cirrhosis

Patients with cirrhosis may be more susceptible to SARS-CoV-2 infection, due to their immunodeficient status, referred as cirrhosis-associated immune dysfunction[79]. In several studies, COVID-19 patients with cirrhosis presented worse prognosis, compared to patients without cirrhosis[80-85]. In a large multicenter study, including 745 COVID-19 patients with CLD (386 with and 359 without cirrhosis), cirrhotic patients exhibited higher mortality rate, compared to those without cirrhosis (32% *vs* 8%, $P < 0.001$)[82]. Mortality was correlated with the stage of liver cirrhosis; 19% in Child- Pugh class A, 35% in class B, and 51% in class C. A similar trend was also observed in the rates of ICU admission, mechanical ventilation and renal replacement therapy. In the same study, it was noted, that the main cause of death was respiratory failure (71%) followed by liver complications[82].

Moreover, COVID-19 patients with cirrhosis are in increased risk for acute decompensation and acute-on-chronic liver failure (ACLF)[78]. Sarin *et al*[86], conducting a multicenter cohort study, found that 20% of patients with compensated cirrhosis developed acute decompensation or ACLF during COVID-19 disease, while 57% of patients with decompensated cirrhosis had further liver complications. Acute decompensation is a common clinical feature in cirrhotic patients during SARS-CoV-2 infection, usually presented as new or worsening ascites or hepatic encephalopathy[82]. Interestingly, liver complications can also be developed and in the absence of typical symptoms of respiratory system[82,85].

Non-alcoholic fatty liver disease

Patients with NAFLD usually have other comorbidities, such as diabetes mellitus, obesity, hypertension and chronic cardiac disease, which are common risk factors for severe COVID-19[77]. Consequently, it is challenging to define an independent effect of NAFLD on COVID-19 and evidence from concomitant studies is controversial. More particularly, some studies did not prove an association between NAFLD and worse COVID-19 outcomes[87-89]. On the other hand, numerous observational studies demonstrated that NAFLD is related to more severe SARS-CoV-2 infection, while three meta-analyses

confirmed this association[90-99]. Despite multiple confounding factors, NAFLD was considered as an independent risk factor for severe COVID-19. Hashemi *et al*[91] found that NAFLD was an independent risk factor for ICU admission and mechanical ventilation in COVID-19 patients. In a retrospective case-control study, NAFLD was associated with COVID-19 severity, irrespective of metabolic syndrome[90]. Furthermore, Sachdeva *et al*[97], in their pooled analysis, reported that NAFLD was a predictor of COVID-19 severity, even after adjusting for obesity.

Alcoholic liver disease

Although the existing evidence is limited, few studies demonstrated that ALD is related to increased COVID-19 mortality. In a multicenter cohort study of 867 COVID-19 patients, reported that ALD is an independent risk factor of higher mortality[100]. Likewise, Marjot *et al*[82] identified independent association between ALD and COVID-19 mortality. Mallet *et al*[101], also, found that ALD is a risk factor of day-30 mortality after COVID-19. The exact mechanism leading to the aforementioned correlation is not clear. However, ALD-related immune dysregulation and low nutritional status may have a negative impact on the course of SARS-CoV-2 infection[7,79].

Viral hepatitis

The influence of viral hepatitis on COVID-19 severity and COVID-19-related liver injury has not been clearly established. COVID-19 patients with chronic hepatitis B (CHB) may have prolonged virus shedding and infection[48]. Furthermore, during SARS-CoV infection, replication of hepatitis B virus (HBV) was found to be enhanced, inducing more severe liver injury; similar enhancement could be noted during SARS-CoV-2 infection[102]. Wang *et al*[103], in a retrospective cohort study of 437 patients, found that those with co-infection SARS-CoV-2/HBV had higher risk of severe disease and mortality. Likewise, Zou *et al*[104] reported that COVID-19 patients with CHB and liver injury were more prone to poor outcomes. Nevertheless, other studies did not demonstrate the above associations. Chen *et al*[105] found no difference in terms of liver function and disease severity between COVID-19 patients with HBV and those without co-infection. Guan *et al*[106] also suggested that CHB does not affect COVID-19 outcome, as only one of 23 patients with CHB developed severe disease. In addition, Yip *et al*[107] demonstrated that current and past HBV infection were not related to higher risk of liver injury or mortality.

Due to extended use of immunosuppressive drugs for COVID-19 treatment (*i.e.*, tocilizumab), potential re-activation of HBV should be taken into consideration. Although the immunosuppressive therapies are short-term and results of clinical trials are contradictory, there are some clinical case reports of HBV re-activation in COVID-19 patients after administration of these immunosuppressive agents[108].

Of note, COVID-19 pandemic has disrupted the progress in the global hepatitis C virus (HCV) elimination program, resulting in delays in diagnosis and HCV therapy, which could extend the direct COVID-19-related morbidity and mortality in these patients[109].

Autoimmune liver disease

Although immunosuppressive therapy, used in patients with autoimmune liver diseases, could be associated with higher risk of severe disease, there is no evidence that patients with autoimmune hepatitis (AIH), primary biliary cholangitis (PBC) or primary sclerosing cholangitis (PSC) are more prone to SARS-CoV-2 infection[6,77]. In a phone-based survey, there was no difference in percentage of COVID-19 diagnosis in patients with autoimmune liver diseases and the general population. Most of patients reported a favorable disease outcome in the same survey[110]. Data derived from three multinational registries (SECURE-Cirrhosis, COVID-Hep and ERN RARE-LIVER) revealed that patients with AIH had increased risk of hospitalization compared to patients with other CLD, but there was no difference in adverse outcome, including ICU admission and death, despite the immunosuppressive treatment[111]. However, a recent retrospective study of 254 patients with COVID-19 and AIH demonstrated that baseline treatment with corticosteroids or azathioprine was associated with COVID-19 severity[112]. Evidence for patients with PBC and PSC is limited and no defined association with COVID-19 severity has been established yet[7].

Hepatocellular carcinoma

COVID-19 patients with may have a high risk for poor outcomes. Due to chemotherapy/immunotherapy, HCC patients are immunosuppressed, and, subsequently, vulnerable to severe SARS-CoV-2 infection[102]. Furthermore, most HCC patients have an underlying CLD (*i.e.*, cirrhosis, ALD *etc.*), and as a result, they are already identified as a high-risk group[102]. However, the corresponding literature is limited. A small retrospective study of 28 cancer patients with COVID-19, including 2 HCC patients) found that these patients had worse prognosis compared to general population[113].

VACCINATION AGAINST SARS-COV-2 IN CHRONIC LIVER DISEASE

Different types of SARS-CoV-2 vaccines have been developed, such as mRNA vaccines, adenoviral-vectored vaccines and inactivated vaccines. In general, patients with CLD may exhibit lower immune response to vaccination; according to previous studies, rate of seroconversion after HBV vaccine and cell-mediated immunity were reduced in cirrhotic patients[114,115]. Regarding efficacy, although trials of both mRNA vaccines included few patients with underlying CLD, they reported significant efficacy in the subgroup with coexisting comorbidities[116,117]. Of note, in a large retrospective cohort study of cirrhotic patients, a single mRNA vaccine dose appeared to reduce not only rates of SARS-CoV-2 infection, but also, rates of hospitalization and mortality[118]. With regard to safety, none of vaccine contain living virus, and subsequently, they can be used even in immunosuppressed patients[119]. Moreover, no significant liver-associated side effects have been reported in the vaccinated population [120]. Given that benefits outweigh the potential risks, European Association for the Study of the Liver (EASL) and American Association for the Study of Liver Diseases (AASLD) recommend that patients with CLD should be vaccinated against SARS-CoV-2[121,122].

MANAGEMENT OF LIVER INJURY IN COVID-19

Liver injury in COVID-19 is usually mild and resolves spontaneously without any special treatment [77]. If present, hypoxia and circulatory failure should be regulated with standard symptomatic support (*i.e.*, oxygen therapy, intravenous fluids) in order to prevent further liver damage[45,123]. If liver injury persists, underlying chronic liver disease should be suspected[124]. With regard to DILI, there are no specific management guidelines. Discontinuation or dose's reduction of suspected medication is the most effective treatment in case of DILI, as the only available antidote is N-acetylcysteine for acetaminophen overdose[67]. In the case of severe COVID-19, benefits and risks have to be weighed in order to decide discontinuation of systematic treatment. This dilemma hardly arises for pharmaceutical agents which need short administration, such as remdesivir and tocilizumab[67]. Standard guidelines and supportive therapy should be followed for management of acute liver failure[67].

Regarding chronic liver diseases, comprehensive recommendations related to COVID-19 management have been published by EASL-ESCMID and AASLD[124,125]. Cirrhotic patients with acute decompensation or ACLF have to be tested for COVID-19, even without any other symptom[125]. Patients with HBV or HCV and SARS-CoV-2 coinfection should continue antiviral therapy, while in COVID-19 patients with chronic, occult or resolved HBV, who receive immunosuppressive agents (*i.e.*, tocilizumab, corticosteroids), clinicians have to consider and prevent potential HBV re-activation[124,125]. In COVID-19 patients with AIH, discontinuation or reduction of immunosuppressive agents is not recommended. Reduction is considered in special cases, such as severe COVID-19 and bacterial/fungal co-infection, or severe lymphopenia[124,125].

CONCLUSION

Liver abnormalities are common in COVID-19 patients, especially in patients with severe and critical disease. The pathogenesis of liver injury may be multifactorial involving direct cytopathic viral effect, inflammatory storm, hypoxic/hypoperfusion injury and drug hepatotoxicity. Liver injury is usually mild and transient; however, some cases of severe liver injury and acute liver failure have been reported. Although, patients with stable chronic liver disease are not more vulnerable to SARS-CoV-2 infection, patients with cirrhosis, ALD, NAFLD and HCC have higher risk for severe COVID-19 and liver damage. Specific management issues should be taken into consideration during COVID-19 treatment in patients with underlying CLD. Further investigation is needed in order to clarify the association between SARS-CoV-2 and liver dysfunction, in terms of prognosis, pathophysiology and treatment.

FOOTNOTES

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Country/Territory of origin: Greece

ORCID number: Ioanna Papagiouvanni 0000-0001-5190-0154; Ioannis Goulis 0000-0002-2765-4157.

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REVIEW

Advancing the precision management of inflammatory bowel disease in the era of omics approaches and new technology

Xin-Yu Liu, Hao Tang, Qing-Yang Zhou, Yan-Lin Zeng, Dan Chen, Hui Xu, Yue Li, Bei Tan, Jia-Ming Qian

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Xin-Yu Liu, Qing-Yang Zhou, Yan-Lin Zeng, Hui Xu, Yue Li, Bei Tan, Jia-Ming Qian, Department of Gastroenterology, Peking Union Medical College Hospital, Peking Union Medical College & Chinese Academy of Medical Science, Beijing 100730, China

Xin-Yu Liu, Eight-year Medical Doctor Program, Peking Union Medical College & Chinese Academy of Medical Science, Beijing 100730, China

Hao Tang, Department of Internal Medicine, Peking Union Medical College Hospital, Peking Union Medical College & Chinese Academy of Medical Science, Beijing 100730, China

Yan-Lin Zeng, School of Medicine, Tsinghua University, Beijing 100084, China

Dan Chen, Department of Gastroenterology, Beijing Hospital, National Center of Gerontology, Institute of Geriatric Medicine, Chinese Academy of Medical Science, Beijing 100730, China

Corresponding author: Bei Tan, MD, Associate Professor, Department of Gastroenterology, Peking Union Medical College Hospital, Peking Union Medical College & Chinese Academy of Medical Science, No. 1 Shuaifuyuan, Dongcheng District, Beijing 100730, China. tanbei0626@aliyun.com

Abstract

There is great heterogeneity among inflammatory bowel disease (IBD) patients in terms of pathogenesis, clinical manifestation, response to treatment, and prognosis, which requires the individualized and precision management of patients. Many studies have focused on prediction biomarkers and models for assessing IBD disease type, activity, severity, and prognosis. During the era of biologics, how to predict the response and side effects of patients to different treatments and how to quickly recognize the loss of response have also become important topics. Multiomics is a promising area for investigating the complex network of IBD pathogenesis. Integrating numerous amounts of data requires the use of artificial intelligence.

Key Words: Inflammatory bowel diseases; Precision management; Multiomics; Artificial intelligence

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Core Tip: Inflammatory bowel diseases (IBDs) exhibit different pathogeneses and clinical manifestations. Making precise and appropriate therapeutic decisions according to the condition of each patient remains challenging. We summarize the clustering strategies, the approaches used to apply multiomics and artificial intelligence to IBD precision management.

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INTRODUCTION

Since it was first proposed in 2011[1], the concept of precision medicine has become increasingly popular and attracted much attention. Great progress has been made, especially in the treatment of cancer. Precision medicine typically refers to the use of targeted therapy based on etiology and mechanism. The essence of the idea involves classifying individuals with common characteristics into the same subgroup using specific clinical features, treatment features and prognoses. Thus, this strategy should actually combine a wide array of data, including clinical, genetic and environmental information, as well as multiple types of biomarkers[2]. These efforts would add to the objectivity and flexibility of treatment decision-making.

Inflammatory bowel disease (IBD) is a group of intestinal disorders of unknown etiology characterized by inflammation that arises from a complex interaction between genetic and environmental factors and immune responses[3]. An increasing number of studies have reported on the great heterogeneity of IBD patients in terms of pathogenesis, clinical manifestation, response to treatment and prognosis, and IBD is currently regarded as a continuous spectrum of diseases[4]. The introduction of biologics has greatly improved the quality of life of IBD patients, which also embodies precision medicine to some extent. However, due to the complexity of pathogenesis, targeting only immune pathways without addressing the genome or microbiome may result in limited success[5]. The treatment strategies used are largely based on evidence from clinical trials, which typically do not stratify patients with enough precision. Additionally, the frequency of treatment may be indicated for a certain population, but this approach might not be the most suitable for an individual. Compared to the oncology field, there is still much room for precision medicine development in IBD.

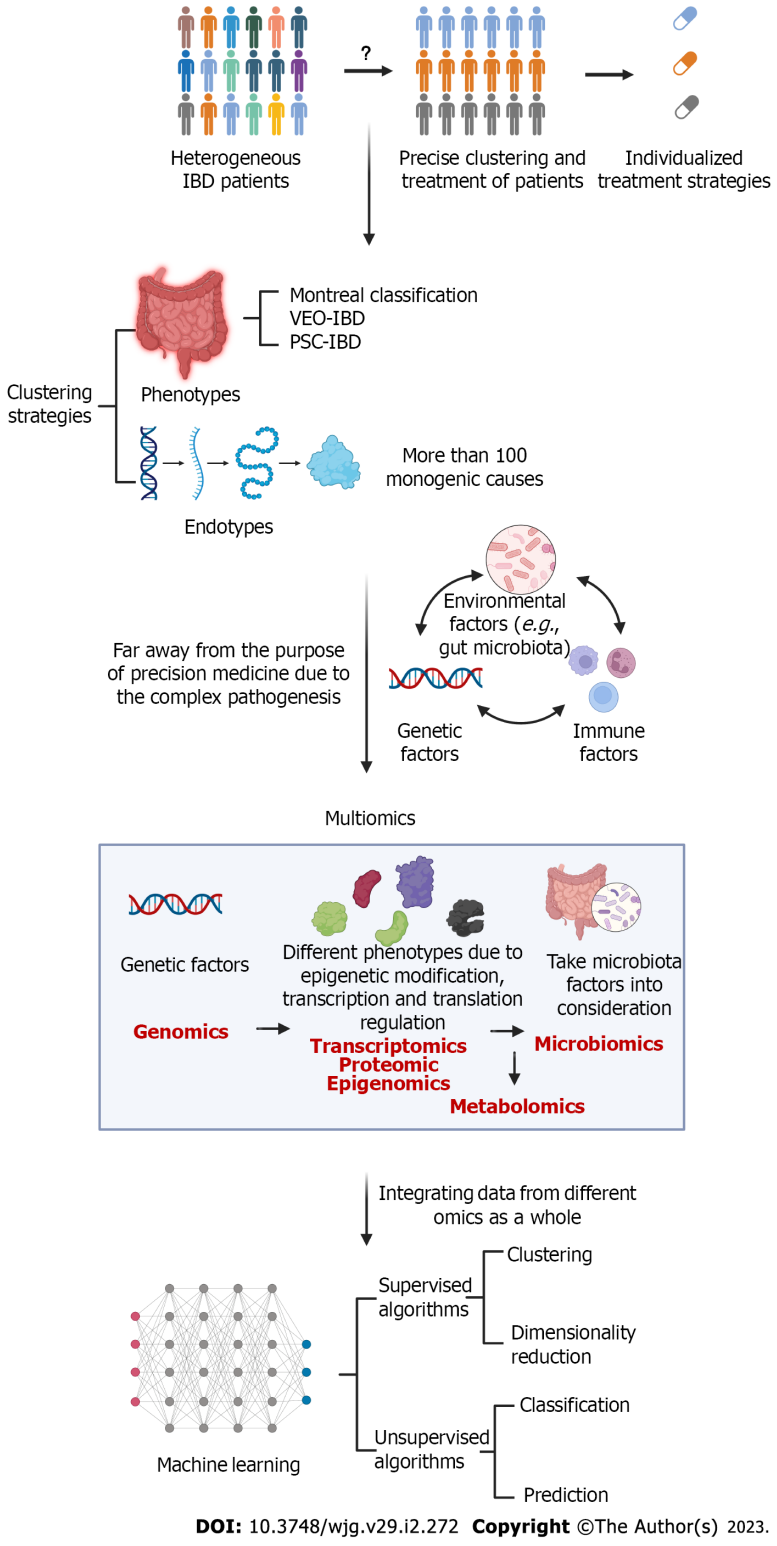
In this review, we discuss the strategies used to categorize IBD patients and biomarkers for identifying these subgroups. We suggest that applications of multiomics and artificial intelligence (AI) approaches could facilitate the precision management of IBD patients (Figure 1).

THE HETEROGENEITY OF IBD AND CLUSTERING STRATEGIES FOR IBD PATIENTS

Phenotype refers to the traits that can be observed in patients, and deep phenotyping plays a key role in the progress of precision medicine[2]. In other disease contexts, there is also the concept of endotype, which is defined as the molecular mechanism underlying the visible phenotype[6]. However, clustering phenotypes and endotypes remains difficult in the context of IBD due to heterogeneity.

The Montreal classification is the most widely used clinical classification of IBD and considers age at diagnosis, location and behavioral factors[7]. The characteristics and natural history of IBD seemed to vary depending on the age of onset[8]. Very early onset IBD (VEO-IBD), defined as IBD occurring in those who are diagnosed under the age of 6 years and sometimes exhibiting a more aggressive disease pattern, seemed to have stronger genetic triggers with less environmental influence[9]. In addition, complications or extraintestinal organ involvement can also be used to identify some unique subsets of IBD patients. For example, IBD patients who experienced complications with primary sclerosing cholangitis (PSC) have been shown to exhibit higher rates of colectomy, cancer and death than non-PSC-IBD patients[10]. However, current clinical classification is far from the precise identification of IBD phenotypes.

Some specific genetic factors have been found to determine disease progression, which is difficult to assess by clinical manifestations. Next-generation sequencing has been used to identify more than 100 monogenic causes that could manifest as IBD-like phenotypes. The genes involved in monogenic IBD disorders are generally classified into six categories according to the mechanisms: Epithelial barrier defects; T-cell and B-cell defects; hyperinflammatory and autoinflammatory disorders; phagocytic defects; immunoregulation defects; and others[11]. For example, mutations in the IL-10 pathway could lead to neonatal or infantile VEO-IBD with severe enterocolitis and crissum disease by impairing IL-10-



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Figure 1 Flow gram of the precision management strategies of inflammatory bowel disease. IBD: Inflammatory bowel disease; PSC: Primary sclerosing cholangitis; VEO: Very early onset.

mediated control of inflammatory responses involving IL-1 and IL-23. Mutations in *CYBB* could lead to chronic granulomatous disease characterized by intestinal inflammation and autoimmune disease due to impaired antimicrobial activity caused by defects in NADPH oxidase[12,13]. Conventional treatment in patients with the subsets of IBD that are largely driven by genetic factors often exhibit unsatisfactory efficacy, and these patients have poor prognosis. These various mechanisms that underlie the effects of monogenic mutations also have some crossover with the mechanisms involved in sporadic and multifactorial IBD, which reflects the divergence and convergence of the mechanisms. For precision treatment of IBD, strategies should not be limited to therapies targeting upstream etiology; therapy

based on more superficial mechanisms should also be pursued.

The etiology of monogenic causes, which account for only a small percentage of IBD cases, is complex, but sporadic IBD involves many more factors. More than 260 risk loci have been identified to be associated with sporadic IBD by genome-wide association studies (GWASs)[14], yet these loci only explained approximately 20% of the genetic heritability in complex adult-onset IBD[15]. This finding is easy to understand because there are also environmental, microbiota or other factors involved in the pathogenesis of IBD. IBD cannot be classified by a single factor, but the application of biomarkers can aid in the advancement of the precision management of IBD to some extent.

BIOMARKERS

Due to the rising incidence of IBD and the inconvenience of endoscopy, there is an urgent need for noninvasive, accessible and cost-efficient biomarkers. Precision medicine should cover the whole management process of IBD patients, including the early identification of patients at potential risk for disease progression and enabling appropriate adjustments in response to ongoing assessments of treatment efficacy. Such a strategy should be a highly sophisticated process, not just the endpoint of a single stratification approach[16]. Accordingly, we reviewed two categories of biomarkers (mainly from serum and feces) for IBD: Those used to identify disease progression risk and activity and those used to predict treatment responses.

Identifying disease progression risk and activity

C-reactive protein (CRP) is the most widely used serum biomarker for inflammation in IBD[17]. It reflects both clinical disease activity and endoscopic inflammation in IBD patients[18]. Additionally, the level of CRP is not influenced by treatments and thus is also suitable for monitoring treatment response [19]. However, it is not a specific biomarker, and its levels might be elevated in other diseases, including noninflammatory conditions. Additionally, up to 25% of Crohn's disease (CD) patients with endoscopically proven activity could not be identified by CRP[20]. Fecal calprotectin (FC) is another important noninvasive biomarker widely used in clinical practice. In the assessment of endoscopically defined disease activity in IBD, FC analysis exhibits higher sensitivity in the context of both ulcerative colitis (UC) and CD, especially in UC[21,22]. In particular, FC analysis could be used in the early prediction of relapse risk 6 and even 12 mo in advance[23]. A meta-analysis reported 78% sensitivity and 73% specificity when using FC at remission to predict IBD relapse, with cutoffs varying between 120 μg/g and 340 μg/g[24]. However, this biomarker also faces the problem of limited specificity; inflammation in the gut that is not associated with IBD, such as during infection, necrotizing enterocolitis and drug-induced enteropathy, could confound the results[25]. For patients with borderline FC levels, combining FC analysis with other metrics, such as clinical activity indices or CRP levels, could help the assessment [26]. The levels of serum calprotectin (SC), as an indirect marker of inflammatory activity in UC[27], can indicate the involvement of other extraintestinal organs. Another well-accepted fecal biomarker is fecal lactoferrin (FL), the levels of which could also reflect IBD activity and be used to predict disease relapse. Unlike the analysis of FC levels, the advantage of FL is its specificity[28], and combined analysis of these biomarkers might result in better assessment. In addition, some secondary biomarkers measured using simple laboratory tests, such as the CRP-albumin ratio, neutrophil-lymphocyte ratio (NLR), platelet-lymphocyte ratio (PLR) and lymphocyte-monocyte ratio (LMR), can also be used to infer the activity of IBD[29,30].

In recent years, an increasing number of novel serum and fecal biomarkers with application potential have been discovered. The levels of leucine-rich glycoprotein, a glycoprotein that is also related to IBD activity, could be elevated in patients with normal CRP levels during the active period of UC[31]. Some serum antibodies resulting from autoimmunity and loss of immune tolerance to microbial antigens have been considered in the diagnosis and assessment of IBD[32]. For example, anti-*Saccharomyces cerevisiae* antibody (ASCA) and perinuclear anti-neutrophil cytoplasmic antibody (pANCA), which are antibodies of microbial antigens and autoantibodies, respectively[33], are two extensively studied antibodies with high specificity for IBD[34]. They could help identify potential CD patients five years before diagnosis when combined with the analysis of other protein markers[35]. In addition to enabling diagnosis, a higher ASCA titer was related to more aggressive fibrosis and stenosis and internal penetrating disease behaviors[36], while the pANCA titer changed with the activity of UC[37]. In addition, cytokines such as granulocyte colony-stimulating factor were associated with endoscopically active disease[38], while IL-6 and IL-2 Levels could also be used to predict the course of disease relapse 12 mo in advance in quiescent CD patients[39]. Circulating noncoding RNAs, including microRNAs (miRNAs) and long ncRNAs (lncRNAs), also play a role in IBD, and the analysis of miRNAs might help monitor disease activity and stricture phenotypes[32]. Other newly emerging biomarkers for disease progression risk and activity include cathelicidin[40], trefoil factor 3[41], and 25-hydroxyvitamin D3[42]. Several extracellular matrix (ECM) components and growth factors are important biomarkers indicating intestinal fibrosis and stenosis[32]. The analysis of fecal biomarkers, cytokines and other indicators of inflammation could also help with the identification of IBD activity[43]. Fecal myeloperoxidase, another

biomarker related to neutrophil inflammation in addition to FC, was recently reported to accurately indicate endoscopic activity in IBD and predict the disease course during follow-up[44]. However, all these novel biomarkers are mainly used in research and remain far from clinical use (Table 1).

Predicting treatment responses

The mainstream therapeutic drugs for IBD include aminosalicylates (ASAs), glucocorticoids (GCs) and immunosuppressive agents[45]. The treatment of IBD has greatly advanced since the recent introduction of biologics, including tumour necrosis factor-α (TNF-α) inhibitors (such as infliximab and adalimumab), integrin inhibitors (such as vedolizumab and etrolizumab) and IL-12/IL-23 inhibitors (such as ustekinumab). However, selecting among these therapies largely depends on the clinical characteristics, comorbidities, and patients' preferences or concerns, and this decision-making process lacks a uniform objective standard for indicating the path of treatment. Thus, effective biomarkers for predicting treatment responses are urgently needed.

For 5-ASA, a multicenter prospective cohort study in pediatric UC patients developed a predictive model with initial clinical activity and early treatment response to 5-ASA to predict long-term corticosteroid-free remission[46]. The baseline FC level and UC endoscopic index of severity could be used to predict the early outcome of GCs treatment[47].

Clinical responses to biologics are even more varied. However, the application of biologics is usually tried in a certain order by experienced physicians without effective biomarkers used to influence the selection of different kinds of biologics, which is of concern in research. Some laboratory test results are taken into consideration. For example, a clinical trial revealed that using CRP and FC levels in combination with clinical symptoms could result in better clinical and endoscopic outcomes than considering only clinical symptoms[48]. These downstream indicators of active inflammation could suggest a response to anti-inflammatory TNF-α inhibitors, but they are still not enough for accurate prediction of the likelihood of remission in a given patient in a real clinical environment[49]. Thus, we need to explore more biomarkers that could reveal the molecular heterogeneity of patients treated with different biologics.

Existing biologics can be briefly classified into two groups according to underlying mechanisms: inhibitors of cytokines and inhibitors of lymphocyte migration. TNF-α is considered to be a downstream inflammatory pathway effector in multiple immune-related diseases[50]. It is rational to speculate that IBD patients with increased TNF-α levels might have a good response to anti-TNF-α agents. Detecting membrane-bound TNF (mTNF) by endoscopy with the aid of fluorescence labeling has been used to successfully predict the efficacy of anti-TNF-α treatment[51]. Another study also reached this goal by measuring the TNF production capacity of peripheral blood mononuclear cells (PBMCs)[52]. A lack of response to anti-TNF-α therapy might indicate the activation of other inflammatory pathways. Baseline levels of serum oncostatin M, a member of the IL-6 family that might mediate inflammation in another manner, have been reported to be elevated in anti-TNF-α nonresponders and could be used to predict the efficacy of this treatment[53,54]. In addition, low triggering receptor expressed on myeloid cells 1 (TREM1) expression in both whole peripheral blood samples and intestinal biopsy samples, which indicated a complete macrophage autophagy pathway, could be used to predict a good anti-TNF response in IBD patients[55]. Antibodies including anti-drug antibodies (ADA), pANCA and anti-OmpC (*Escherichia coli* outer membrane porin) were also found to be associated with the response to infliximab[56]. In the aspect of relapse after discontinuation of anti-TNF therapy, mucosal TNF gene expression and IL1RL1-transcripts might play a role[57].

The IL23/Th17 pathway is also a central cytokine pathway involved in IBD in addition to TNF-α. The levels of IL-22 and IL-17, the downstream factors involved in this pathway, are potential molecular predictors of the response to IL-23 blockers[58]. Another category of biologics for IBD is integrin inhibitors, which act by inhibiting gut-selective lymphocyte homing. The most widely used integrin inhibitor, vedolizumab, works by blocking the binding of the α4β7 integrin heterodimer on lymphocytes to MAdCAM1 on the gut[59]. Higher expression levels of α4β7 on T, B, and NK cells as well as the presence of $\alpha4\beta7^+$ intestinal mucosa cells could be used to predict responses to vedolizumab[60,61]. However, the predictive role of serum α4β7, VCAM-1 and ICAM-1 remains controversial[62,63]. In addition, higher IL-6 and IL-8 Levels have been reported to be associated with the response to vedolizumab[64] (Table 2).

Sometimes, simply by assessing early responses to biologics, we can judge the potential future efficacy to some extent[65]. Therapeutic drug monitoring (TDM) is another tool for the assessment of biologic therapeutic outcomes based on the findings that drug concentrations correlate with biologic efficacy. However, due to the long time required for detection and the lack of an instructive reference range, there is still no consensus for the use of TDM[66,67].

FUTURE OF PRECISION MANAGEMENT IN IBD: MULTIOMICS

In addition to simple serum and fecal biomarkers, emerging high-throughput analytical technologies offer opportunities for the improved management of IBD. Omics strategies, often including genomics,

Table 1 Biomarkers of serum or feces identifying disease progression risk and activity

Sample	Biomarker	Outcome	Characteristic
Serum	CRP[17-20]	Monitor disease activity and mucosal healing	Widely used and low-cost; lack of specificity for intestinal inflammation; relatively low sensitivity
	SC[27]	Disease burden, prognosis, and relapse	More representative of systemic inflammation
	LRG[31]	Monitor disease activity and mucosal healing	More correlated to activity in UC than CRP
	Serum antibodies		
	ASCA[35,36]	More aggressive fibro stenosing and internal penetrating disease behaviors	CD specificity
	pANCA[35,37]	UC disease activity	UC specificity
	Cytokines		
	G-CSF, IL-1Ra, PDGF-BB[38]	Endoscopically active disease	-
	IL-6, IL-2[39]	Predict disease relapse in quiescent CD	-
	Noncoding RNAs[32]	Monitor disease activity and stricture phenotypes	-
	ECM components[32]	Intestinal fibrosis and stenosis	-
	Growth factors[32]	Intestinal fibrosis and stenosis	-
	Cathelicidin[40]	Mucosal disease activity in UC, risk of intestinal stricture in CD, and clinical prognosis in IBD	-
	Trefoil Factor 3[41]	Monitor disease activity	-
	Vitamin D[42]	Disease activity	-
	Secondary biomarkers (CRP-albumin ratio, NLR, PLR, LMR)[29,30]	IBD activity	Easy to obtain; fluctuates greatly
Feces	FC[21-26]	Monitor disease activity and mucosal healing; early prediction of relapse risk	Higher sensitivity than CRP; confounding of non-IBD gut inflammation
	FL[28]	Monitor disease activity and predict disease relapse	Higher specificity than fecal calprotectin
	MPO[44]	Endoscopic activity in IBD and predict the disease course during follow-up	-

ASCA: Anti-*Saccharomyces cerevisiae* antibody; CD: Crohn's disease; CRP: C-reactive protein; ECM: Extracellular matrix; FC: Fecal calprotectin; FL: Fecal lactoferrin; G-CSF: Granulocyte colony-stimulating factor; IL-1Ra: Interleukin 1 receptor antagonist; IBD: Inflammatory bowel disease; LMR: Lymphocyte-monocyte ratio; LRG: Leucine-rich alpha-2 glycoprotein; MPO: Myeloperoxidase; NLR: Neutrophil-lymphocyte ratio; pANCA: Perinuclear anti-neutrophil cytoplasmic antibody; PDGF-BB: Platelet-derived growth factor BB; PLR: Platelet-lymphocyte ratio; SC: Serum calprotectin; UC: Ulcerative colitis.

transcriptomics, proteomics, metabolomics, epigenomics and microbiomics, have completely transformed the trajectory of medicine. These different omics approaches might also provide some insights into IBD from different perspectives.

Genomics

Genomics aims to characterize and quantify all the genetic information of an organism. Numerous variations in factors of genetic susceptibility involved in many complex diseases have been identified. Different genes have been reported to associate with IBD severity and activity. *NOD2/CARD15* is the most classic CD-related gene found in Western countries[68,69] and is also associated with stricturing behaviors and the need for operation[70,71]. However, it was not found to be related to CD development in East Asian cohorts[72]. Regarding UC, a GWAS developed a risk score based on 46 single nucleotide polymorphisms to identify medically refractory UC that needed colectomy[73]. Regarding therapeutic efficacy, HLA-DQA1*05 carriage was reported to be associated with ADAs for TNF-α inhibitors in CD and suggested the need for combination therapy[74]. In addition, polymorphisms in *TLR2*, *TLR4*, *TLR9*, *TNFRSF1A*, *IFNG*, *IL6*, *IL1B*, *TNF-α* and apoptosis-associated genes (Fas ligand and caspase-6) were also potential genetic biomarkers for the anti-TNF treatment response[75-77]. HLA-DQA1-HLA-DRB1, nudix hydrolase 15 and thiopurine-S-methyltransferase variants were

Table 2 Biomarkers predicting treatment responses

Type of agent	Biomarker	Sample	Outcome
5-ASA	Initial clinical activity, early treatment response of 5-ASA[46]	-	Predict long-term corticosteroid-free remission
GCs	FC, UCEIS[47]	Feces	Predict the early outcome of GCs treatment
Biologics			
TNF-α inhibitors	mTNF[51]	Endoscopy	Predict anti-TNF-α efficacy
	TNF production capacity of PBMCs[52]	Blood	Predict anti-TNF-α efficacy
	OSM[53,54]	Serum	Upregulate in anti-TNF-α non-responders
	TREM1 expression[55]	Blood and intestinal biopsies	Predict anti-TNF-α efficacy
	Antibodies: ADA, pANCA, anti-OmpC [56]	Serum	Associate with the response to infliximab
	Mucosal TNF gene expression and IL1RL1- transcripts[57]	Intestinal biopsies	Predict long-term remission after discontinuation of anti-TNF-α therapy
IL-23 inhibitors	IL-22, IL-17[58]	Serum	Predict anti-IL-23 efficacy
Integrin inhibitors	α4β7 on T, B, and NK cells[60,61]	Blood and endoscopy	Predict responses to vedolizumab
	α4β7, VCAM-1, ICAM-1[62,63]	Serum	Remain controversial
	IL-6, IL-8[64]	Serum	Associate with the response to vedolizumab

ADA: Anti-drug antibodies; ASA: Aminosalicylates; FC: Fecal calprotectin; GCs: Glucocorticoids; ICAM-1: Intercellular adhesion molecule-1; mTNF: Membrane-bound tumour necrosis factor; OmpC: *Escherichia coli* outer membrane porin; OSM: Oncostatin M; pANCA: Perinuclear anti-neutrophil cytoplasmic antibody; PBMC: Peripheral blood mononuclear cell; UCEIS: Ulcerative Colitis Endoscopic Index of Severity; VCAM-1: Vascular cell adhesion molecule-1; TREM1: Triggering receptor expressed on myeloid cells 1.

found to be associated with thiopurine-related adverse events[53]. However, the utility of genomics alone is limited due to the complexity of IBD pathogenesis.

Transcriptomics/proteomics/epigenomics: From genes to phenotypes

In exploring the pathogenesis of complex diseases such as IBD, the limitations of using a single genomics approach are becoming increasingly apparent. The same gene variation might lead to distinct phenotypes by epigenetic modification and transcriptional and translational regulation. Transcriptomics, proteomics and epigenomics could better reflect the gene expression profiles, which combine genetic and environmental factors and thus perform better than simple genomics.

In particular, numerous transcriptomic studies have provided insights into the prediction of IBD progression. Researchers found that the expression of ECM accumulation-associated genes in the ileum was associated with stricturing behaviors in pediatric CD patients. After combining age, race, disease location, and antimicrobial serology factors, they established a competing-risk model that reached a specificity of 71%[78]. However, ileum samples are difficult to access, which poses a barrier to the utility of this approach. Studies on blood samples are thus warranted. Transcriptional profiling of circulating $CD8^+$ T cells successfully distinguished patients with a risk of aggressive disease mainly based on the expression of genes involved in T-cell responses[79]. Furthermore, this classification was also feasible for use in whole blood samples with transcriptional signatures based on 17 genes[80]. The transcriptional risk score, which represented the summation of risk alleles for CD from ileum or blood samples, could be used to identify patients who would progress to complicated disease[81]. Regarding treatment, UC patients could be clustered into different groups with distinct transcriptomic profiles of the rectum and showed different responses to anti-TNF therapy[82].

Unlike transcriptomics, the use of proteomics in the context of IBD is still in its infancy. Some studies have sought to detect proteins involved in early inflammatory mechanisms of IBD, and some proteome analyses have been performed in studies with small sample sizes to investigate the differentiation of disease behavior as well as the prediction of response to biological treatment[83,84]. In the Proteomic Evaluation and Discovery in an IBD Cohort of Tri-service Subjects (PREDICTS) study, a series of protein biomarkers involved in the complement cascade, lysosomes, innate immune response, and glycosaminoglycan metabolism along with some antibodies were able to be used to predict potential CD patients 5 years in advance[35]. Another study revealed that MMP10, CXCL9, CXCL11, and MCP1 were upregulated in UC patients before disease onset[85]. However, there is still a long way to go before these approaches are applied clinically.

Epigenetic mechanisms mainly include DNA methylation, histone modifications, and noncoding RNAs. The complement of methylated DNA in the genome is called the methylome. Epigenetic alterations have been detected when IBD patients were compared with healthy individuals[86]. The number of epigenomic studies investigating IBD subgroup identification is still limited. The earliest finding observed in this area involved the assessment of cancer risk in the context of UC[87]. In future research, epigenomics studies might provide useful biomarkers for the early detection of cancer development in UC patients.

Microbiomics: Perspective of the environment

All of the above strategies provide omics analysis of the host. As mentioned previously, the gut microbiota, as an environmental factor, also plays an important role in the pathogenesis of IBD. Due to the convenience of fecal sample collection, microbiomics is promising for monitoring and managing IBD patients. The microbiota might also be able to be used to predict relapse risk. For example, a deficit in some bacterial groups or species, such as *Faecalibacterium prausnitzii* and *Bacteroides*, could be a predictive factor for relapse of CD after ileal resection or infliximab cessation[88,89]. Another study revealed that *Ruminococcus* and *Veillonella* were associated with stricturing and penetrating complications, respectively[78]. A recent prospective study classified CD patients into different subgroups with different clinical relapse risk based on microbiota[90]. Additionally, microbial analysis revealed distinct microbiota compositions between patients with different responses to anti-TNF-α therapy[84,91] as well as anti-integrin therapy[92]. Furthermore, manipulation of the microbiota might be a direction for IBD treatment.

However, this method is easily influenced by environmental factors such as diet and confounded by the causal relationship between microbiota and IBD; thus, its reliability is questionable. Recent findings are still at a superficial stage of providing simple differences in microbial abundance, and there has been a lack of in-depth analysis of microbial networks and microbiota-host interactions, as well as solid and effective prediction models.

Metabolomics

Metabolomics generally includes serum and fecal metabolomics. As a combination of host metabolic factors and environmental gut microbiota factors, it is also a potential technique for use in future IBD research and clinical practice. Several studies have applied metabolic profiling for the diagnosis and classification of IBD[32]. A serum metabolomics study identified altered lipid and amino acid metabolism in parallel with CD activity[93]. Short-chain fatty acids (SCFAs) have been widely validated to be beneficial metabolites[94], among which butyrate is one of the most important. Studies have confirmed that fecal SCFA levels were reduced during active IBD[95]. Butyrate levels were associated with the efficacy of azathioprine, TNF-α inhibitors and integrin antibodies[92,96,97]. Other metabolites, such as bile acids and tryptophan, are also worth studying for future use[94].

APPLICATION OF ARTIFICIAL INTELLIGENCE FOR INTEGRATED OMICS

Due to the complexity of IBD pathogenesis, interpretation of a single set of omics data often fails to provide insight into complex biological phenomena; thus, the omics approaches discussed above must be considered as a whole. Integrating multiple omics strategies into a network would contribute to the elucidation of the pathway involved in pathogenesis and facilitate the identification of different subgroups and the optimization of therapy regimens in IBD. The analysis of different molecules, including at the genomic, transcriptomic, proteomic, microbiome, epigenetic and metabolomic levels, could be performed simultaneously, and the results could be further integrated into multiomics models [98]. By this approach, we could obtain more insight into disease pathogenesis, identify more promising predictive biomarkers and facilitate early diagnosis[99]. Some multiomics projects are ongoing and are investigating IBD heterogeneity to improve precision management[53].

These high-throughput data need to be modeled by AI algorithms with the aid of advanced computational techniques. Machine learning is a subset of AI where machines can learn from experience provided by the data without the need for programming. Machine learning includes supervised and unsupervised algorithms. Supervised algorithms are often used for classification or prediction using example data, while unsupervised algorithms are often used for clustering according to similarity[100]. These approaches could be well applied to address the need for patient clustering and predictions and the detection of novel biomarkers. Progress made in machine learning has benefited the integrated analysis of multiomics data; these strategies mainly include concatenation-, model- and transformation-based methods[101]. In addition, deep neural networks have been used in the integration of multiomics data for the prediction of drug efficacy in cancer therapy[102], which indicates progress may be made in the context of IBD.

However, due to the obscure nature of machine learning, the robustness of the models established is sometimes uncertain. Thus, testing in independent cohorts and even clinical trials are needed before this approach is employed in a clinical setting. Additionally, products that are easy to implement in clinical

settings need to be developed from research.

CONCLUSION

The pathogenesis of IBD remains uncertain, which challenges the clustering and precision management of patients. Genetic, environmental and immune factors are all involved in the complex process of IBD development. Thus, the future direction of IBD management may largely rely on the development of multiomics analysis. Numerous data processing workflows require the help of AI.

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Country/Territory of origin: China

ORCID number: Xin-Yu Liu 0000-0003-3991-2245; Qing-Yang Zhou 0000-0002-3205-8579; Hui Xu 0000-0002-8459-7654; Yue Li 0000-0001-6799-1812; Bei Tan 0000-0002-0053-7399; Jia-Ming Qian 0000-0001-6570-9262.

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REVIEW

Screening and interventions to prevent nonalcoholic fatty liver disease/nonalcoholic steatohepatitis-associated hepatocellular carcinoma

Simona Cernea, Danusia Onișor

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Simona Cernea, Department M3/Internal Medicine I, George Emil Palade University of Medicine, Pharmacy, Science and Technology of Târgu Mureş, Târgu Mureş 540139, Romania

Simona Cernea, Diabetes, Nutrition and Metabolic Diseases Outpatient Unit, Emergency County Clinical Hospital, Târgu Mureş 540136, Romania

Danusia Onişor, Department ME2/Internal Medicine VII, George Emil Palade University of Medicine, Pharmacy, Science, and Technology of Târgu Mureş, Târgu Mureş 540139, Romania

Danusia Onişor, Gastroenterology Department, Mureş County Clinical Hospital, Târgu Mureş 540072, Romania

Corresponding author: Simona Cernea, MD, PhD, Associate Professor, Department M3/Internal Medicine I, George Emil Palade University of Medicine, Pharmacy, Science and Technology of Târgu Mureş, 38 Gheorghe Marinescu st., Târgu Mureş 540139, Romania.
simonacernea@yahoo.com

Abstract

Liver cancer is the sixth most commonly diagnosed cancer worldwide, with hepatocellular carcinoma (HCC) comprising most cases. Besides hepatitis B and C viral infections, heavy alcohol use, and nonalcoholic steatohepatitis (NASH)-associated advanced fibrosis/cirrhosis, several other risk factors for HCC have been identified (*i.e.* old age, obesity, insulin resistance, type 2 diabetes). These might in fact partially explain the occurrence of HCC in non-cirrhotic patients without viral infection. HCC surveillance through effective screening programs is still an unmet need for many nonalcoholic fatty liver disease (NAFLD) patients, and identification of pre-cirrhotic individuals who progress to HCC represents a substantial challenge in clinical practice at the moment. Patients with NASH-cirrhosis should undergo systematic HCC surveillance, while this might be considered in patients with advanced fibrosis based on individual risk assessment. In this context, interventions that potentially prevent NAFLD/NASH-associated HCC are needed. This paper provided an overview of evidence related to lifestyle changes (*i.e.* weight loss, physical exercise, adherence to healthy dietary patterns, intake of certain dietary components, *etc.*) and pharmacological interventions that might play a protective role by targeting the underlying

causative factors and pathogenetic mechanisms. However, well-designed prospective studies specifically dedicated to NAFLD/NASH patients are still needed to clarify the relationship with HCC risk.

Key Words: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Hepatocellular carcinoma; Risk stratification; Lifestyle interventions; Prevention

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Core Tip: Nonalcoholic fatty liver disease (NAFLD) is a public health problem, especially in developed countries. This condition, depending on certain associated risk factors, can ultimately lead to liver cirrhosis and hepatocellular carcinoma (HCC). Having the necessary tools and knowing the characteristics of patients in whom the disease progresses more quickly, effective monitoring programs can be developed. Primary prevention of NAFLD/nonalcoholic steatohepatitis (NASH)-associated HCC relies on controlling the main modifiable risk factors. Some pharmacological (*e.g.*, metformin, statins, aspirin) and non-pharmacological interventions (weight loss, physical exercise, healthy diet, avoiding heavy drinking and smoking) might have protective effects. Herein, we emphasized the need for continued investigations to find the optimal methods for NAFLD/NASH-associated prevention.

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INTRODUCTION

Primary liver cancer was estimated to be the fourth leading cause of cancer-related deaths and the sixth most commonly diagnosed cancer in 2018 worldwide, with hepatocellular carcinoma (HCC) comprising most cases (75%-85%)[1]. The main risk factors for HCC are chronic infection with hepatitis C virus (HCV) and hepatitis B virus (HBV), and non-viral factors, such as heavy alcohol drinking or nonalcoholic fatty liver disease (NAFLD)/nonalcoholic steatohepatitis (NASH) and associated metabolic disorders [like type 2 diabetes mellitus (T2DM), obesity], which have emerged as important determinants of the disease[2]. In fact, while the incidence and mortality rate related to viral-associated HCC is decreasing lately, NAFLD/NASH has become a major cause of cirrhosis and HCC[3]. This is relevant considering the increased prevalence of NAFLD, which affects about one-quarter of the adult population worldwide[4]. Even if the HCC risk is lower in NAFLD patients compared to the HCV-infected patients, it is still seven times higher in comparison with the general population[5,6].

Besides NASH/advanced fibrosis and cirrhosis, several other risk factors for HCC have been identified (Figure 1). Among these, T2DM appears to be strongly and independently associated with both NAFLD/NASH and HCC[7]. In fact, the prevalence of NAFLD in patients with T2DM is twice as high, and the prevalence of NASH is about seven-ten times higher, while the risk of HCC is 2.0-2.5 fold higher than in the general population[4,8,9]. Some studies suggest that a longer duration of diabetes can increase the risk of HCC[10]. The underlying mechanisms that link T2DM to HCC are complex and not fully elucidated, but insulin resistance, chronic inflammation, lipotoxicity, and oxidative stress may play a substantial role by promoting DNA damage, angiogenesis, cellular growth and proliferation, and decreasing cellular apoptosis[11-13]. In fact, insulin resistance seems to play an important role in HCC development (through associated proinflammatory, vasoactive, and pro-oxidative environment), and it might explain in part the occurrence of HCC in non-cirrhotic NAFLD patients[6,13].

Although not unanimous, the overall evidence is suggestive of an increased risk of HCC in individuals with obesity [as evaluated by the body mass index (BMI)][7,14,15]. A case-control study performed in the United States has identified obesity in early adulthood (mid-20s to mid-40s) as a significant HCC risk factor [odds ratio (OR): 2.6, 95% confidence interval (CI): 1.4-4.4], as each unit of increased BMI was associated with a 3.89-mo decrease in age of HCC diagnosis ($P < 0.001$)[16]. Moreover, obesity in childhood (ages 7-13-years-old) was reported to be associated with higher HCC risk later in life in a retrospective cohort comprising 285884 Danish children[17]. Visceral obesity appears to be particularly significant as an HCC risk, regardless of BMI[18,19]. The gut-liver axis seems to play an important role in the obesity-associated HCC, as gut microbiota creates a tumor-promoting microenvironment by transferring its metabolites/components, which further trigger the release of proinflammatory cytokines (like tumor necrosis factor alpha, interleukin-1β, interleukin-6, *etc.*), suppress the anti-tumor immunity, and modify the bile acid metabolism[20-22].

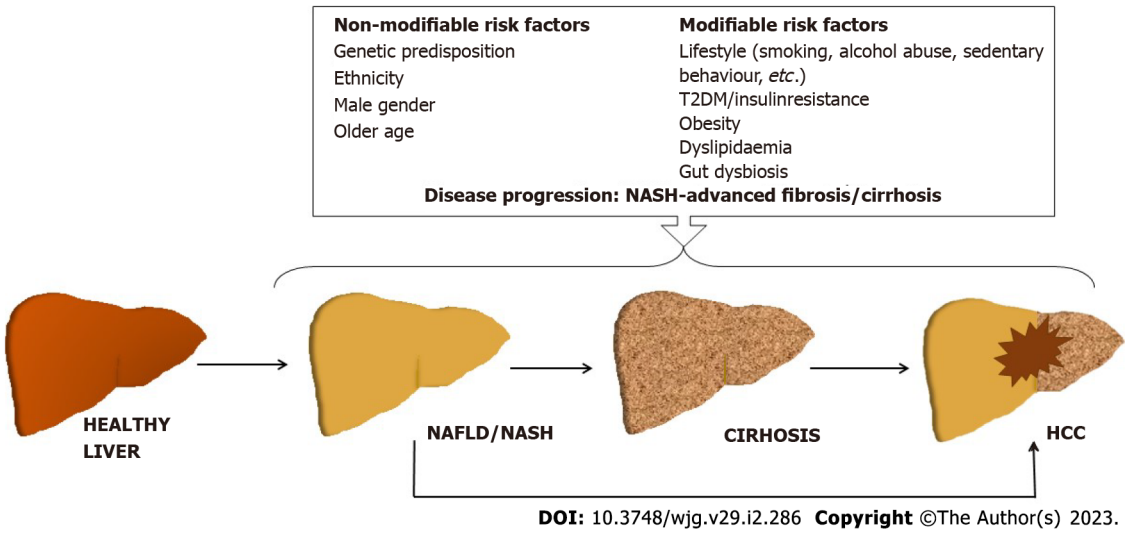


Figure 1 Risk factors for nonalcoholic fatty liver disease/nonalcoholic steatohepatitis -associated hepatocellular carcinoma. HCC: Hepatocellular carcinoma; NAFLD: Nonalcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; T2DM: Type 2 diabetes mellitus.

Other risk factors for NAFLD-related HCC are male sex, older age, smoking, genetic predisposition (*i.e. PNPLA3* polymorphism, *etc.*) (Figure 1)[6,23-27]. In addition, dyslipidemia, ethnicity, intestinal dysbiosis, and sedentary lifestyle may also contribute (Figure 1)[3,6,26,28-30]. Apparently, the presence of multiple risk factors increases the risk of liver cancer synergistically[6].

In fact, multiple hits drive the development of the NAFLD/NASH-associated HCC through activation of various metabolic, endocrine, and immunological pathways (*i.e.* increased free fatty acids levels/impaired lipid metabolism, hyperinsulinemia, oxidative stress, endoplasmic reticulum stress, and hyperleptinemia and increased production of proinflammatory cytokines, altered immune response, release of pro-fibrinogenic mediators, *etc.*) on a background of genetic/epigenetic alterations [31].

RISK STRATIFICATION AND PREDICTION

The goal of HCC surveillance in NAFLD/NASH patients is to reduce the HCC-related mortality by promoting early tumor detection[32]. Controversy still exists regarding which NAFLD patients would benefit most from the HCC surveillance[6]. In NAFLD patients, the risk of liver-related and all-cause mortality raises exponentially with higher fibrosis stage (from F1 to F4)[33]. The meta-analysis by Dulai *et al*[33] indicated that compared to F0, the rate ratio of the all-cause mortality was 1.58 (in stage 1), 2.52 (in stage 2), 3.48 (in stage 3), and 6.40 (in stage 4), and the same trend was seen for liver-related mortality. However, it should be noted that the evolution of fibrosis is not linear, as it progresses and regresses in about 20%-30% of patients over 5 years[34]. Among patients with NAFLD, those with cirrhosis are at greatest risk, with an annual HCC incidence rate of 10.6/1000 person-years (PY) compared to 0.08/1000 PY in patients without cirrhosis[5]. Furthermore, HCC incidence rates are higher in patients with decompensated cirrhosis than in those with compensated cirrhosis[28].

Nevertheless, NAFLD patients without cirrhosis are still at risk of developing HCC. The analysis of data obtained from a cohort of 1500 patients with HCC showed that about 13% of them did not have cirrhosis, and patients with NAFLD had a five-fold increased risk of developing HCC in the absence of cirrhosis compared with those with HCV-related HCC[35]. A lower proportion of patients with NAFLD-associated HCC presented cirrhosis than patients with HCV- or alcohol-related HCC (58% *vs* 85.6% and 72.4%, respectively)[35]. The same was basically shown by the meta-analysis by Tan *et al*[36] (61 studies; 94636 patients). They reported that NAFLD-related HCC patients were more likely to be non-cirrhotic (38.5% *vs* 14.6%, $P < 0.0001$) and had larger tumor diameters ($P = 0.0087$)[36]. Moreover, these patients had undergone surveillance in a lower proportion than patients with HCC secondary to other causes (32.8% *vs* 55.7%, $P < 0.0001$)[36].

Poor HCC surveillance is a significant problem for patients with NAFLD, and in fact, identification of pre-cirrhotic NAFLD individuals with high HCC risk remains a significant challenge at the moment. A prospective multicenter study performed in Italian secondary care centers that included 756 patients with NAFLD- or HCV-related HCC has shown that HCC was diagnosed though regular ultrasound/specific surveillance in a lower proportion of NAFLD patients compared to HCV patients (47.7% *vs* 63.3%, $P < 0.0001$), resulting in a more advanced HCC burden at diagnosis in the former group[37]. Similarly, the analysis of data from the United States Veterans Administration HCC cohort study

showed that more patients with NAFLD-HCC did not benefit from HCC surveillance 3 years before diagnosis (43.3%) compared to patients with alcohol abuse- or HCV-related HCC (40.2%, and 13.3%, respectively)[38].

Solid data and guidance regarding risk stratification in non-cirrhotic NAFLD patients who might benefit from HCC surveillance are limited, and specific recommendations in this area are urgently needed due to the growing epidemic of NAFLD[39].

How to perform the screening?

Liver biopsy remains the "gold standard" for the diagnosis of NASH, but it cannot be routinely used in practice as a screening method to diagnose NAFLD, given its multiple limitations: It is expensive, the procedure is subject to interpretation errors, and it is potentially associated with adverse effects such as pain, bleeding, and infection[40,41]. The emergence of non-invasive methods for quantifying fibrosis and their validation has led to a decrease in the need for liver biopsies[42-44]. The Asia-Pacific and the American Gastroenterology Association guidelines agree that the combined use of serum tests and imaging tools may provide more reliable information than using either method alone[30,45]. The American Association for the Study of Liver Diseases guideline also consider the non-invasive methods as first-line tests for the investigation of fibrosis, but it does not recommend a specific diagnostic algorithm or follow-up strategies[43].

Identification of fibrosis and risk stratification is an essential step for HCC surveillance, as the guidelines clearly recommend screening in patients with cirrhosis, while patients with advanced fibrosis (F3) might also undergo surveillance based on individualized evaluation.

Currently, the primary imaging method for HCC detection is ultrasound (US)[40,46,47]. However, recent studies have highlighted the limitations of this examination[28]. For example, a study comprising 941 patients with cirrhosis who underwent ultrasonography reported that 20% of examinations had an inadequate quality to exclude images showing possible focal points[48]. Therefore, other methods (computer tomography, magnetic resonance imaging) might be used[45,47]. For these two investigations, the follow-up interval is not clearly established nor are the benefits of the association with measurement of alpha-fetoprotein (AFP) levels[49].

It has been questioned whether the addition of AFP quantification to routine biannual US examinations would increase the detection rate of HCC during screening. However, only about three-quarters of HCC patients are AFP positive[50]. In fact, the probability of having elevated AFP levels (*i.e.* > 10 ng/mL) in patients with early NAFLD-associated HCC without cirrhosis and normal transferase levels was only 17.5%-24.0% compared with 86.5%-90.5% in patients with viral-associated cirrhosis and advanced HCC with increased transaminases values, as showed in an Italian study that included 4123 HCC patients[51]. The use of AFP measurement as a screening tool for HCC might result in earlier diagnosis, but it does not seem to improve the mortality rate[52]. The European guidelines do not currently recommended AFP as a surveillance parameter for the detection of HCC in patients with NAFLD. The American guidelines recommend ultrasound screening with or without AFP, while Asia Pacific Society guidelines recommend screening with AFP[30,47,53]. Other biomarkers such as microRNAs (*e.g.*, miR-34a, miR-221) are under investigation for early detection of HCC but need further validation[54].

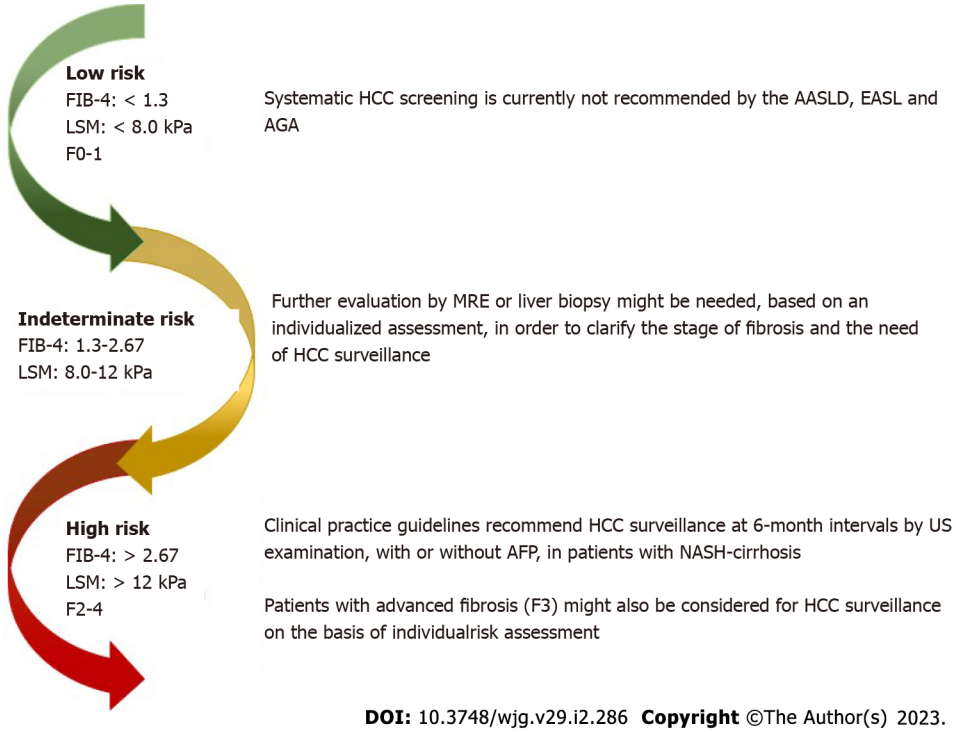
HCC screening in low risk NAFLD-patients

Most patients screened in a primary care setting have a low risk of clinically significant liver fibrosis, defined as having a Fibrosis-4 (FIB-4) score < 1.3, liver stiffness measurement (LSM) < 8.0 kPa on transient elastography, or a liver biopsy fibrosis stage of F0–F1[44,55-57]. Systematic HCC screening may not be prudent and is currently not recommended by the American Association for the Study of Liver Diseases, American Gastroenterology Association, and the European Association for the Study of the Liver in non-cirrhotic NAFLD patients (Figure 2)[42-44].

HCC screening in indeterminate risk-NAFLD patients

An estimated 30%–40% of screened patients have an indeterminate risk of clinically significant (advanced) liver fibrosis, defined as FIB-4 score of 1.3-2.67 and/or a LSM of 8.0-12.0 kPa on transient elastography[44,55-57]. The estimated incidence of HCC in non-cirrhotic NASH seems to be too low to justify systematic screening[58]. Apparently, non-cirrhotic NAFLD patients with multiple features of metabolic syndrome are at higher risk of HCC development and need special attention[5,35]. It is argued that additional triggers (such as active inflammation and fibrosis) are needed to promote carcinogenesis[6].

The current guidelines recommend the referral of these patients to a hepatologist for further evaluation by magnetic resonance elastography or liver biopsy[30,42,43]. The decision should be taken by mutual agreement and on the basis of an individual assessment (presence/absence of comorbidities, degree of fibrosis, *etc.*)[44]. The European Association for the Study of the Liver guidelines recommend that patients with liver disease and advanced fibrosis (F3) might be considered for HCC surveillance based on the individual risk, while the Asian Pacific and American Association for the Study of Liver Diseases clinical practice guidelines do not provide a specific recommendation for surveillance in



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Figure 2 Recommended hepatocellular carcinoma screening approach in nonalcoholic fatty liver disease/nonalcoholic steatohepatitis patients according to the risk category. AASLD: the American Association for the Study of Liver Diseases; AFP: Alpha-fetoprotein; AGA: American Gastroenterology Association; EASL: The European Association for the Study of the Liver; FIB-4: Fibrosis-4; HCC: Hepatocellular carcinoma; LSM: Liver stiffness measurement; NASH: Nonalcoholic steatohepatitis; MRE: Magnetic resonance elastography; US: Ultrasonography.

patients with NAFLD without cirrhosis[30,43].

HCC screening in high risk-NAFLD patients

Nearly 10% of screened patients have a high risk of advanced liver fibrosis, defined as a FIB-4 score > 2.67, LSM > 12.0 kPa, or a liver biopsy showing clinically significant liver fibrosis (≥ F2)[44,55-57]. Patients with cirrhosis are at the highest risk for HCC. The meta-analysis and meta-regression by Orci *et al*[59] involving 470404 patients showed that the incidence rate of HCC was 0.03/100 PY in patients with NAFLD at a pre-cirrhotic stage and 3.78/100 PY in those with cirrhosis, while in patients with cirrhosis undergoing regular screening for HCC, it was 4.62/100 PY.

Some data suggested that HCC surveillance might not be associated with improved clinical outcomes. For example, a matched case-control study from the United States Veterans Affairs health system failed to find an association between screening (by US, AFP, either test, or both tests) and rate of HCC-related mortality[60]. However, the lack of benefit may have not been related to the failure of surveillance but rather to other causes, such as underuse of HCC treatment or applying surveillance in patients who were not candidates for HCC treatment. On the other hand, a meta-analysis of 59 cohort studies indicated that HCC surveillance was associated with improved early HCC detection, receiving curative therapy, and survival in patients with cirrhosis but with heterogeneity in pooled estimates[32]. Thus, available data is in favor of HCC surveillance in patients with cirrhosis, although it still needs further confirmation[32].

Surveillance programs by regular US (and AFP) in patients with compensated cirrhosis are cost effective[61]. In fact, cost-effectiveness analyses indicate that HCC screening should be considered for patients with Child-Pugh A and B (compensated) cirrhosis and decompensated liver cirrhosis patients waiting for liver transplantation[47].

In patients with NASH-cirrhosis, all three liver study societies recommend the use of an HCC surveillance program at 6-mo intervals, with US exams, with or without AFP[30,42,43]. The same was endorsed by the recommendations of American Gastroenterology Association Clinical Practice[40].

INTERVENTIONS TO PREVENT NAFLD/NASH-ASSOCIATED HCC

The NAFLD/NASH-associated HCC primary prevention relies on controlling the main modifiable risk factors, *i.e.* obesity, T2DM/insulin resistance, gut dysbiosis, disease activity/fibrosis (disease progression), that have been associated with activation of various oncogenic pathways finally leading to

hepatocarcinogenesis[62]. There is no clearly effective intervention for HCC prevention available for NAFLD/NASH patients at the moment, although some pharmacological and non-pharmacological approaches might indeed be useful by addressing the predisposing factors/causes and underlying pathogenetic mechanisms (Table 1)[63].

Weight loss and bariatric surgery

Weight loss through lifestyle intervention represents the cornerstone for NAFLD management, as it has been associated with the regression of steatosis, steatohepatitis, and even fibrosis (for > 10% weight loss) [64]. The analysis of two randomized controlled trials (RCTs) indicated that each lost kg was associated with a 7% increase in odds of obtaining NASH resolution without worsening of fibrosis, and a 5% increase in odds of obtaining fibrosis improvement without worsening of NASH[65]. The meta-analysis by Koutoukidis *et al*[66], which included 2588 NAFLD subjects who underwent weight loss interventions (through behavioral programs, pharmacotherapy, or bariatric surgery), indicated that these interventions were associated with the improvement of liver steatosis, histologic NAFLD activity score (NAS) and presence of NASH (OR: 0.14, 95%CI: 0.04-0.49) but not of fibrosis.

Preliminary results from a retrospective analysis of a database containing 72 million unique patients reported that weight loss medications reduced the risk of HCC in obese populations (OR: 0.07), with orlistat and liraglutide showing statistically significant decreases (OR: 0.13, and 0.35, respectively)[67]. In addition, the meta-analysis by Ramai *et al*[68], which included data from major databases, indicated that bariatric surgery was associated with a reduced risk of HCC (pooled unadjusted OR: 0.40), although with high heterogeneity (I2: 79%).

Although there is limited evidence regarding the impact of weight loss on the risk of developing HCC, it is intuitive and reasonable to encourage overweight/obese patients to decrease their weight. Weight loss is associated with the improvement of metabolic health (or indirect outcomes, such as the decrease of insulin resistance, inflammation, oxidative stress, *etc.*), which may translate into liver health benefits[69-72]. However, a recent RCT in patients with NAFLD demonstrated that a modest weight loss (-4 kg) through a calory-restricted diet was accompanied by reduction in transaminases, but the liver steatosis grade or the markers of oxidative stress were not significantly changed in comparison with the controls[73]. Thus, the liver benefits still have to be demonstrated by prospective data, which should clarify the direct effect of weight loss on long-term NAFLD/NASH progression and primary HCC prevention.

The effects of bariatric surgery on liver outcomes might be explained in part by weight loss, although other contributing mechanisms cannot be excluded [*e.g.*, increase in glucagon-like peptide-1 (GLP-1) concentrations after intervention][74]. The meta-analysis by Lee *et al*[75], which analyzed the data of 32 cohort studies (n = 3093 liver biopsies from NAFLD patients with obesity that underwent bariatric surgery) showed that surgical intervention resulted in an absolute percentage BMI reduction of 24.98% (from 48.68 ± 2.92 to 34.2 ± 3.53 kg/m^2). This was accompanied by steatosis resolution in 66% of patients as well as the resolution of inflammation (in 50% of patients), ballooning degeneration (in 76%), and fibrosis (in 40%)[75]. However, 12% of the subjects presented new or worsening fibrosis after the intervention[75].

The more recent meta-analysis by Ramai *et al*[68] included nine studies (19514750 patients) and reported that bariatric surgery was associated with a reduced risk of HCC (pooled unadjusted OR: 0.40, 95%CI: 0.28-0.57 and adjusted OR: 0.63, 95%CI: 0.53-0.75). So far there is no clear indication regarding the type of surgical intervention that would be most beneficial in terms of liver health.

Lifestyle changes

A large prospective cohort study has demonstrated the association of unhealthy lifestyle (assessed by a composite score comprising BMI, alternative Mediterranean diet, alcohol intake, smoking, and sleep duration) with the risk of HCC: Higher composite scores (5, 6, 7, 8) representing healthier lifestyle were associated with a lower risk of HCC (0.67, 0.61, 0.49, and 0.13, respectively; $P_{trend} < 0.0001$) compared with lower scores (0-4) over a mean follow-up of 17.7 years[76]. As unhealthy lifestyle is associated with a higher risk of NAFLD/NASH and HCC, it is reasonable to assume that correcting these behaviors will potentially protect against the development of HCC.

However, there is insufficient direct evidence to indicate that changes in lifestyle reduce the risk of HCC in NAFLD/NASH patients. A meta-analysis of 30 RCTs in NAFLD patients (n = 3280), which evaluated the effect of diet, exercise, or their combination on the liver and metabolic markers, reported that a combination of diet and exercise resulted in a greater decrease of ALT [mean difference (MD): -13.27], AST (MD: -7.02) and Homeostatic Model Assessment for insulin resistance (MD: -2.07) compared to either of them[77]. However, no histological or imaging data were available. Moreover, an umbrella review of evidence from observational studies and RCTs looking at the association between lifestyle and NAFLD with regards to risk and treatment (41 meta-analysis from observational studies and 81 meta-analysis from RCTs) suggested that some interventions [*i.e.* green tea, omega 3 polyunsaturated fatty acids (PUFA), and exercise] were associated with some improvement in metabolic and hepatic markers, but more robust RCTs are needed to investigate the effect of lifestyle changes on liver outcomes[78]. In addition, the network meta-analysis by Buzzetti *et al*[79] (59 RCTs, 3631 participants; 2-24 mo of follow-up) could not draw a definite conclusion regarding the effect of the lifestyle interventions on any of the

Table 1 Summary of lifestyle and pharmacological intervention with potential preventive effects against developing nonalcoholic fatty liver disease/nonalcoholic steatohepatitis-associated hepatocellular carcinoma

Interventions with potential protective effects	
Lifestyle interventions	Weight loss
	Dietary changes
	Adherence to healthy eating patterns: Mediterranean diet, traditional Cantonese dietary pattern; Chinese Healthy Eating Index
	Reduced intake of: Saturated fats, sugar-sweetened beverages, alcohol
	Increased intake of: Vegetables, coffee; possibly fiber, white meat and fish, omega-3 polyunsaturated fatty acids, vitamin E
	Physical exercise
	Smoking cessation
Pharmacological interventions	Metformin
	Statins
	Aspirin
	Possibly also: Pioglitazone, GLP-1 RA, vitamin E (in nondiabetic individuals?), obeticholic acid

GLP-1 RA: Glucagon-like peptide-1 receptor agonists.

clinical liver outcomes (including HCC), as the number of events was too low (probably due to short duration of follow-up).

Diet: There is limited information regarding the impact of diet/dietary components on liver histology in patients with NAFLD/NASH and on the risk of progression to HCC. Most studies report data related to liver biomarkers/fat content or risk of liver cancer in overall population/patients with chronic liver diseases, regardless of etiology. The relationship between HCC and several nutrients, foods, and dietary patterns has been evaluated mostly in observational studies, but few data exist exclusively in patients with NAFLD/NASH[80]. There is a lack of high-quality data from large RCTs in this population, but it should be noted that it is quite challenging to evaluate dietary determinants of the disease in the context of multiple confounding effects of other lifestyle factors[81].

Dietary patterns represent a complex combination of foods/nutrients and beverages that are habitually consumed, and their evaluation may capture in a more integrated way the effect of diet on health outcomes[82]. A recent systematic review of 30 observational studies (5222534 participants from Asia, America, and Europe) investigated the association between diet and risk of HCC and found differences with regards to geographical regions and dietary patterns[83]. Specifically, the Mediterranean diet appeared to be protective for the European and American populations, while the Chinese Healthy Eating Index and the Cantonese Dietary Pattern seemed to be associated with lower risk of HCC in Asian countries[83].

Analysis of combined data from two case-control studies demonstrated that better adherence to the Mediterranean diet was associated with a lower risk of HCC (ORs: 0.66 and 0.51, $P < 0.001$ for trend)[84]. In addition, the Alternate Mediterranean diet score (an adaptation of the original Mediterranean diet score) was associated with a decreased risk of HCC [hazard ratio (HR): 0.68; $P = 0.02$], as indicated by a multiethnic cohort prospective study[85]. Another study has shown a suggestive but nonsignificant association with lower risk (HR: 0.75; $P_{trend} = 0.18$)[86]. The Singapore Chinese Health Study data also indicated that higher Alternate Mediterranean diet scores as well as higher scores of Alternative Healthy Eating Index-2010 (AHEI-2010) and Dietary Approaches to Stop Hypertension, representing a better dietary quality, were associated with a lower risk of HCC ($P_{trend} < 0.05$)[76]. The results were in agreement with the report of the National Institutes of Health-AARP Diet and Health study, indicating that higher HEI-2010 and Alternate Mediterranean diet score were significantly associated with lower HCC risk (HR: 0.72; $P_{trend} = 0.03$ and HR: 0.62; $P_{trend} = 0.0002$, respectively)[87].

Similarly, better adherence to the Chinese Dietary Guidelines, as assessed by the Chinese Healthy Eating Index, was shown to be associated with a lower risk of HCC (OR: 0.43; $P < 0.001$), even in the stratified analysis by risk factors[88]. The study by Lan *et al*[89] that enrolled 782 patients with primary liver cancer and evaluated their habitual dietary intake found that an urban prudent dietary pattern (consisting of higher intake of eggs, mushrooms, dairy products, soy foods and nuts, and lower intake of refined grains) and a traditional Cantonese dietary pattern (characterized by a high intake of fruit and vegetables, Cantonese soup, fish, and Chinese herb tea) have been associated with a lower risk of primary liver cancer (OR: 0.25 and 0.61, respectively), while a diet rich in meat and preserved foods

increased the risk (OR: 1.98). Moreover, a prospective study that enrolled 887 patients with newly diagnosed HCC suggested that a higher adherence to the 2016 Chinese Dietary Guidelines was associated with a lower risk of HCC-specific mortality (HR: 0.74) and all-cause mortality (HR: 0.75)[90].

Inflammation is a key pathogenetic mechanism that influences NASH progression and hepatocarcinogenesis. Two studies have evaluated the correlation between dietary inflammatory score/index (that reflect the overall inflammatory potential of a diet) and the risk of HCC/primary liver cancer mortality. The first one showed that higher dietary inflammatory index values (indicating a proinflammatory diet) were associated with an increased risk of liver cancer (HR: 2.05) and liver cancer-associated mortality (HR: 1.97)[91]. The second study reported that higher adherence to empirical dietary inflammatory pattern score (indicating a proinflammatory potential of the diet) was associated with an increased risk of HCC (HR: 2.03; P_{trend} = 0.001)[92]. The same study reported that several other scores (empirical dietary index/lifestyle pattern score for hyperinsulinemia and insulin resistance) indicating a higher insulin resistance potential of a diet were also correlated with a higher risk of HCC[92].

Thus, a healthy dietary pattern (generally characterized by an increased intake of vegetables, fruits, nuts, and whole grains and a decreased intake of red and processed meats) appears to be protective against HCC and HCC-related mortality. However, more specific evaluations are needed to confirm the data and assess the strength of these associations in patients with NAFLD/NASH.

There is little evidence with respect to the impact of dietary macro- and micronutrients upon liver histology in patients with NAFLD/NASH (*e.g.*, reversal of fibrosis) and the risk of progression to HCC.

Data coming from observational studies are heterogenous. Some studies showed that increased carbohydrate intake was associated with higher aminotransferases levels, liver fat, and NASH, while others indicated the opposite or were neutral[81,93-97]. A meta-analysis of ten RCTs concluded that low-carb diets significantly reduced the intrahepatic lipid content but did not change the serum liver enzyme concentrations in patients with NAFLD[98]. There is also sparse and inconsistent evidence regarding the association between food glycemic index and load with HCC[35]. Data from the European Prospective Investigation into Cancer and Nutrition cohort (EPIC) study (477206 subjects) did not find significant associations between dietary glycemic index or glycemic load or total carbohydrate intake with risk of liver cancer[99]. However, the risk of HCC in correlation with types of carbohydrates appeared to be divergent: 43% higher risk per 50 g total sugar intake/d and 30% lower per 50 g starch/d[99]. The analysis of the Shanghai Women's Health Study and the Shanghai Men's Health Study data also indicated no consistent association between dietary carbohydrates, glycemic index, and glycemic load and risk of liver cancer[100].

On the other hand, higher intake of dietary fructose/sugar-sweetened beverages (surrogate for free sugars) has been associated with NAFLD, independent of other risk factors in several studies, as well as with higher intrahepatic lipid content, mainly when consumed in the context of excessive caloric intake [81,101-105]. The meta-analysis by Li *et al*[106] (71 observational studies) reported that higher sugar-sweetened beverages intake was associated with higher overall cancer risk [relative risk (RR): 1.12; P = 0.000] and mortality risk (RR: 1.07; P = 0.029) as well as higher risk of HCC (RR: 2.00; P = 0.001) (but HCC results were based only on two studies). Interestingly, the EPIC study data suggested that consumption of > 6 servings of combined soft drinks per week was associated with higher HCC risk (HR: 1.83; P_{trend} = 0.01) but artificially-sweetened rather than sugar-sweetened soft drinks intake appeared to be deleterious[107]. A significant positive association between carbonated beverages consumption and HCC risk was also seen in a case-control study of 582 cirrhotic patients (181 with HCC) (OR: 2.44; P_{trend} = 0.021)[108].

The same study showed an inverse correlation of HCC risk with fiber intake (OR: 0.49; P_{trend} = 0.012), which is in accordance with the EPIC study results indicating a 30% HCC risk reduction per 10 g/d of total dietary fiber intake, even in viral hepatitis-free individuals[99,108]. However, the analysis of data from two United States cohort studies (125455 participants) did not find a significant association of HCC risk with cereal, fruit, or vegetable fiber intake[109]. On the other hand, lower daily (mainly soluble) fiber intake was observed in patients with NAFLD/NASH in several observational studies[94,96,110].

There is inconsistent evidence with regards to the role of dietary proteins (types, amount) in the progression of NAFLD/NASH and occurrence of HCC. The Rotterdam Study which included 3882 individuals (1337 with NAFLD) showed that animal protein intake was significantly associated with overweight NAFLD after adjustment for metabolic covariates (OR: 1.36)[111]. Indirect evidence, in line with these results, was provided by another cross-sectional study that showed that total and animal protein consumption was positively associated with estimates of liver steatosis (OR: 1.25 and 1.27, respectively), while vegetable proteins had an inverse association with these (OR: 0.81)[112]. On the other hand, a recent RCT that evaluated the effect of a low-carbohydrate high-protein diet on liver fat in 72 T2DM patients demonstrated that it reduced the liver fat content to a slightly greater extent compared to a conventional diet (64% *vs* 51%, P = 0.051) beyond the effects of (similar) weight loss[73]. Similarly, a small prospective study in NAFLD patients with T2DM showed that high protein diets (30% of total caloric daily intake), either animal or plant-based, significantly reduced liver fat independent of change in body weight, and decreased the serum levels of fibroblast growth factor 21[113]. The animal protein diet determined a greater increase in postprandial levels of methionine and branched chain amino acids but this was not accompanied by beneficial or deleterious effects[113]. Some experimental data suggested potential benefits of branched chain amino acids in terms of hepatocarcinogenesis

inhibition (changes in growth factors gene expression, inhibition of proliferation), increased apoptosis in liver cancer cell lines, and improvement of fibrosis[82,114,115]. In addition, few Japanese clinical studies suggested that branched chain amino acid supplementation in patients with HCC may reduce cancer recurrence after hepatic resection (mainly in patients with higher insulin resistance), but others showed no effect[116-119]. Certainly, additional good-quality evidence is needed regarding the role of dietary proteins/amino acids in the progression of NAFLD/NASH and associated HCC.

Most studies concerning dietary fats in NAFLD/NASH and HCC evaluated the role of type rather than amount of fats[120]. Observational studies seem to indicate that total dietary fat intake is not correlated with HCC risk[121-123]. Some but not all results suggested that vegetable fats might be associated with reduced HCC risk[121,123]. Higher consumption of saturated fatty acids however was shown to be associated with hepatic steatosis/NAFLD and NASH[81,95,124]. A large Chinese prospective cohort study also indicated that dietary saturated fats were associated with higher liver cancer risk [adjusted HR (aHR): 1.14; P = 0.005], but results from the EPIC study and a hospital-based case-control study from the United States did not support a direct association of saturated fats intake with HCC[125-127]. However, a meta-analysis of 14 studies by Zhao *et al*[128] demonstrated that higher dietary intake of saturated fats was associated with an increased risk of liver cancer (RR: 1.34 for highest *vs* lowest intake) in a dose-dependent manner.

On the other hand, the same United States case-control study showed that monounsaturated fatty acids (MUFA) but not total PUFA intake was inversely correlated with the risk of HCC (OR: 0.49 and 1.82)[122]. This was in contrast with the study by Yang *et al*[123] that showed that PUFA was inversely associated with HCC risk (aHR: 0.83; P = 0.03) (with MUFA being neutral). The study by Li *et al*[125] indicated that MUFA had a neutral effect (aHR: 1.26, 95%CI: 0.96-1.65; P = 0.034), while PUFA intake was associated with higher HCC risk (aHR: 1.41, 95%CI: 1.07-1.86 for highest *vs* lowest quartile; P = 0.005). Thus, data on MUFA and total PUFA seems quite heterogenous (maybe explained in part by dietary source, type of study, and study population).

In a NASH animal model, dietary intake of docosahexaenoic acid appeared to be superior to that of eicosapentaenoic acid in attenuating Western-diet induced liver injury, oxidative stress and fibrosis, and potentially in reducing the risk of HCC, thus suggesting differential effects of the two dietary omega-3 PUFAs[129]. Evidence is more convergent regarding the effect of omega-3 PUFA supplementation on liver fat content. Two meta-analyses actually showed that it decreased liver fat, and this was confirmed by a small histologic study in non-cirrhotic NASH patients that received 3000 mg/d omega-3 PUFA for 1 year[130-132]. However, the study did not reach the primary end-point (*i.e.* NAS reduction of ≥ 2 points without fibrosis progression)[132].

In addition, the evidence regarding the effect of omega-3 PUFA on HCC risk is not unequivocal. The case-control study by Moussa *et al*[127] demonstrated an inverse association between dietary omega-3 intake and HCC risk (OR: 0.50), but other epidemiological data seemed to indicate a neutral effect (HR: 0.63; P_{trend} = 0.14 and aHR: 0.89, 95%CI: 0.75–1.04; P = 0.14, respectively)[122,123,125]. Some observational studies showed a positive relationship between dietary intake of omega-6 PUFA and HCC risk (adjusted OR: 2.29 for highest *vs* lowest tertile, and OR: 4.36), indicating a potentially negative effect, although other data showed an inverse association (HR: 0.54; P_{trend} = 0.02)[123,127,133]. Linoleic acid intake might be inversely associated with HCC risk (OR: 0.35, P < 0.01)[122].

Even if the role of micronutrients in preventing the progression of NASH to HCC might be hypothetically explained from a pathogenetic perspective (*i.e.* antioxidant, anti-inflammatory, anti-fibrotic effects), and the experimental data is quite consistent. There is insufficient good quality clinical research regarding their dietary intake or supplementation effect in NAFLD patients[134].

Two United States prospective studies have shown an inverse relationship between (dietary and supplemental) magnesium intake and risk of liver cancer (HR: 0.65 and HR: 0.44; P_{trend} = 0.0065, respectively)[135,136]. Results from two large cohort studies in China demonstrated an inverse association between dietary manganese intake and liver cancer risk long-term (HR: 0.51; P_{trend} = 0.001), even after adjustment for HBV infection[137]. The case-control study by Rizk *et al*[108] also found that manganese intake was significantly lower in HCC patients *vs* controls (OR: 0.56; P_{trend} = 0.038) as well as potassium intake (OR: 0.44; P_{trend} = 0.004). On the other hand, the sodium intake was significantly higher [104]. Nevertheless, more studies are needed to clarify the role of minerals in NASH and HCC.

The same above-mentioned case-control study also indicated lower intake of vitamins E and B9 in HCC patients[108]. In the same sense, a report from two Chinese cohort studies showed that dietary vitamin E intake and supplement use were inversely associated with the risk of liver cancer (HR: 0.52) [138]. The effect of therapeutic intervention with vitamin E will be discussed below. An inverse association between HCC risk and (β) carotenes/vitamin A was noted in several studies (with OR: 0.48 for β carotene, 0.34 for vitamin A, and 0.35 for carotenes)[122,139].

Food items/groups have been evaluated with regards to their association with HCC risk. We will only briefly mention the main findings here. Several systematic reviews and meta-analyses have evaluated data regarding the association between meat consumption and risk of HCC. Even if not totally in agreement, they seem to indicate that read meat consumption is associated with increased HCC risk (RR: 1.22) or is neutral (RR: 1.04 and 1.10), and there is an increased HCC risk associated with processed meat consumption (RR: 1.20, and 1.01)[140-142]. Total meat intake had no significant effect [140-142]. On the contrary, white meat and fish intake were found to be inversely associated with the

risk of HCC (RR: 0.69, 0.76 for white meat and RR: 0.78, 0.91 for fish)[141,142]. Some epidemiological data suggested that increased dairy product intake (mainly milk and high-fat diary) was associated with a higher risk of HCC, although not all studies agreed (yogurt seemed to be associated with a decreased risk or had a neutral effect)[121,143-145]. Two meta-analyses indicated that increased vegetable consumption was associated with a lower risk of HCC[146,147]. For other food items, there is insufficiently consistent evidence so far.

There have been suggestions for the use of many herbal and dietary natural compounds, such as prebiotics/polyphenols, resveratrol, and curcumin in NAFLD/NASH therapy, including for HCC prevention, with some small studies suggesting anti-inflammatory effects of prebiotics, but until now there is very limited data from clinical trials, and no clear conclusion can be drawn[134,148-150]. Preclinical data demonstrated the anti-inflammatory effects of the catechin-rich green tea extract that may attenuate NASH-associated liver injury through a decrease of hepatic nuclear factor kappa-B activation but also indirectly through prebiotic and antimicrobial effects on gut microbiota, resulting in decreased translocation of the gut-derived endotoxins[151]. Green tea contains several bioactive compounds that may exert anticarcinogenic properties (*e.g.*, flavonoids, caffeine, polyphenols, *etc.*) through modulation of different signaling transduction and metabolic pathways (reduction of chronic inflammation, oxidative stress, insulin resistance, liver steatosis, *etc.*)[80,152]. The EPIC study data showed an inverse association between tea consumption and HCC risk (HR: 0.41, 95%CI: 0.22-0.78; P_{trend} = 0.003), while a meta-analysis of 15 RCTs demonstrated that green tea reduced the liver enzymes values in NAFLD patients[153,154].

The use of probiotics in NAFLD/NASH, cirrhosis, and HCC has been reported in several studies and indicated that they can improve aminotransferases and insulin resistance and have anti-inflammatory effects, but there is no evidence so far with regards to HCC prevention[150,155]. A meta-analysis of 21 RCTs (1252 participants) suggested that the use of probiotics/synbiotics was associated with a decrease of inflammation markers, liver stiffness, and steatosis in subjects with NAFLD[156]. However, well-designed RCTs are further needed to fully understand their protective effect in patients with NASH.

Coffee: Eight meta-analyses of prospective cohort and case-control studies provided consistent evidence regarding an inverse relationship between coffee drinking and risk of HCC (RR ranging between 0.54 and 0.78), with only one meta-analysis indicating a neutral effect (RR: 0.93)[157-164]. Caffeinated rather than decaffeinated coffee seemed to have a more consistent effect on HCC[158,161]. Also, the association appeared to be related to the amount of daily coffee intake. The beneficial effects generally started at two cups/d. An extra cup of coffee/d reduced the cancer risk by about 15%-25% (RRs between 0.75 and 0.85), and two extra cups/d by about 14%-44% (RRs between 0.56 and 0.86)[157-164].

Alcohol: Alcohol is a major risk factor for HCC, and it has synergistic effects with the metabolic risk factors (T2DM, obesity) in inducing carcinogenic mechanisms[80,165]. Evidence suggests that a modest alcohol intake is protective against fatty liver, NASH, and fibrosis, but it was not firmly established if it is also protective against HCC[82,166]. A meta-analysis of 19 cohorts (4445 incident cases of liver cancer) showed neutral effects of moderate drinking (< 3 drinks/d) on liver cancer risk (RR: 0.91, 95%CI: 0.81-1.02) compared to no drinking, while heavy drinking (defined as ≥ 3 drinks/d) significantly increased the risk (RR: 1.16, 95%CI: 1.01-1.34)[167]. In line with these results, a prospective evaluation of 8345 subjects with hepatic steatosis (mean follow-up duration of 11.1 years) demonstrated a dose-response relationship for advanced liver outcomes/liver cancer that became significant at ≥ 10 g of alcohol intake/d (for liver outcomes) and ≥ 30 g/d (for liver cancer) after multivariate adjustments[168]. The decrease in risk of HCC after alcohol drinking cessation is uncertain, but a meta-analysis of four studies suggested a decline of HCC risk with 6%-7%/year, although caution was advised in data interpretation [169].

Physical activity: The benefits of physical activity in reducing insulin resistance and hepatic liver content in NAFLD patients are well known[170,171]. The meta-analysis by Golabi *et al*[170] (eight studies with 8 to 48 wk duration) reported that aerobic and resistance exercises determined a liver fat reduction of 30.2%.

Physical exercise intervenes at multiple levels in the pathogenic pathways of NAFLD by reducing the free fatty acid (FFA) flux to the liver, FFA hepatic synthesis, the mitochondrial and cellular damage, oxidative stress, damage-associated molecular patterns release, and hepatic stellate cell activation and by increasing the FFA oxidation and activating the AMP-activated protein kinase-regulated pathways, *etc.*[172]. Physical exercise also improved mitochondrial function (*i.e.* autophagy, biogenesis) and modulated the carcinogenic signaling pathways[80,173,174]. Taken together, these might explain the potential protective effects of exercise in NAFLD/NASH and HCC. Indeed, a meta-analysis of 14 prospective studies indicated that physical activity was inversely correlated with the risk of liver cancer (HR for high *vs* low physical activity: 0.75, 95%CI: 0.63-0.89)[175]. These results were in accordance with the EPIC study that reported an aHR of 0.55, 95%CI: 0.38–0.80 for HCC in active *vs* inactive individuals [176]. The associations seemed to be at least in part mediated by obesity[176]. However, no prospective study evaluated the effect of physical exercise on HCC risk, as this might be rather difficult to perform [172].

Smoking cessation: Data from the Liver Cancer Pooling Project, a consortium of 14 prospective cohort studies comprising 1518741 individuals indicated that cigarette smoking significantly increased the risk of HCC (HR: 1.86, 95%CI: 1.57–2.20)[177]. It also demonstrated that in individuals who quit smoking for > 30 years, the risk of HCC decreased to values almost similar to non-smokers (HR: 1.09, 95%CI: 0.74–1.61)[177]. In addition, quitters seemed to have a lower risk of HCC-related mortality (HR, 0.62, 95%CI: 0.39-0.97), but this was seen in subjects without diabetes[178].

Pharmacological interventions

Several drugs have been suggested to bring benefits for NAFLD/NASH and to potentially reduce the risk of associated HCC, although there is limited evidence for HCC chemoprevention. It is assumed however that by improving histological features associated with NASH and the primary drivers of fibrogenesis ultimately leading to cirrhosis (and HCC), the disease progression will be attenuated, and HCC occurrence eventually prevented[179].

Metformin: Metformin does not seem to improve NASH/fibrosis, but several meta-analyses suggested that it might reduce the risk of HCC in patients with T2DM[180-182]. A recent meta-analysis of 24 studies including 1.4 million individuals reported that metformin was associated with a 41% lower risk of HCC in DM patients treated with metformin ($P < 0.001$) and a significant reduction of all-cause mortality (HR: 0.74, 95%CI: 0.66-0.83; $P = 0.037$)[180]. Moreover, a network meta-analysis that compared several antidiabetic medications has shown that in comparison with sulphonylureas and insulin, metformin significantly decreased the risk of HCC (RR: 0.45, 95%CI: 0.27–0.74 and RR: 0.28, 95%CI: 0.17–0.47)[181]. Postulated mechanisms of chemoprotective effects of metformin are the activation of AMP-activated protein kinase and inhibition of the mammalian target of rapamycin pathway, the inhibition of angiogenesis and induction of apoptosis[183]. It was also suggested that metformin may inhibit the progression of high fat diet-induced HCC by modulating the innate immune-mediated inflammation and restoring tumor surveillance[184]. However, these data should be interpreted with caution, and there is still need for further substantiation.

Thiazolidinediones: The meta-analysis by Musso *et al*[185] has delineated the effects of the two thiazolidinediones (TZDs), indicating that only pioglitazone (30/45 mg/d, for 6 to 24 mo) was associated with improvement in fibrosis (even advanced fibrosis) and NASH resolution in patients with or without diabetes. In addition to increasing adiponectin levels and decreasing excessive lipolysis and FFA flux into the liver, pioglitazone attenuated oxidative stress and inflammation and, the activation and proliferation of hepatic stellate cells, extracellular matrix deposition, fibrosis, *etc*[12,186-188]. The possible role of the TZDs in hepatic chemoprevention is further suggested by *in vitro* data, showing that they may inhibit hepatocarcinogenesis by the regulation of nucleophosmin, a ubiquitously expressed cellular phosphoprotein involved in both proliferation and growth-suppression pathways[189,190]. Animal data also showed that pioglitazone reduced the HCC development, possibly through the upregulation of the AMP-activated protein kinase pathway and the reduction of the mitogen-activated protein kinase activation[191]. However, data in humans are scarce, and no definite conclusions can be drawn yet. The results of the network meta-analysis by Zhou *et al*[181] suggested possible beneficial effects of TZDs in reducing HCC incidence, but these were seen only in comparison with sulphonylureas (RR: 0.47, 95%CI: 0.22–0.97) and with insulin (RR: 0.30, 95%CI: 0.14–0.61), but not *vs* observation alone.

GLP-1 receptor agonists: Two histological studies with GLP-1 receptor agonists (GLP-1 RAs) (liraglutide and semaglutide) in patients with or without T2DM have proven NASH resolution, but results regarding fibrosis were inconclusive[192,193]. Preclinical studies have suggested potential chemoprotective effects of the GLP-1 RAs through various mechanisms like enhancing natural killer cell-mediated cytotoxicity and, inducing autophagy and senescence of the HCC cells by the increase of transforming growth factor β1, promoting their apoptosis though activation of the JNK signaling pathway, *etc*[194-196]. There is very limited information regarding the long-term effect of GLP-1 RAs on HCC incidence in humans so far.

Statins: Two recent meta-analyses of observational and interventional studies have confirmed liver safety for statin use in patients with NAFLD and even a reduction of transaminases levels[197,198]. Moreover, the meta-analysis by Fatima *et al*[198], which also analyzed the liver histology outcomes, reported a significant reduction of steatosis grade, necro-inflammatory stage, and of significant fibrosis but not the fibrosis stage. Several meta-analyses (mostly of observational studies) have consistently reported reduced risk of HCC in statin users (RRs/ORs/HR between 0.52-0.75 for all statins), with some indication of differences between them[199-208]. In particular, it seemed that the lipophilic statins exert preventive effects (OR: 0.51, 95%CI: 0.46-0.57 and HR:0.49, 95%CI: 0.39–0.62) rather than the hydrophilic statins (OR: 0.77, 95%CI: 0.58-1.02 and HR: 0.73, 95%CI: 0.40–1.34)[205,208]. Higher doses appeared to be associated with better protective effects (HR: 0.38 *vs* 0.55)[195]. Moreover, a meta-analysis of nine retrospective cohort studies also reported a lower risk of HCC-related mortality (RR: 0.78, $P = 0.001$) and reduced HCC recurrence (RR: 0.55, $P < 0.001$) with statin use[208]. However, another meta-analysis

indicated that statin usage decreased the risk of all-cause mortality (HR: 0.80, 95%CI: 0.68-0.94; $P < 0.0001$) but not of HCC-specific mortality (HR: 0.80, 95%CI: 0.62-1.03; $P = 0.002$)[200].

Apart from exerting lipid-lowering effects, statins have anti-inflammatory, antioxidant, anti-proliferative, and anti-angiogenic properties[12,209,210]. Their potential anti-tumor effects might be mediated through the downregulation of the RAF/mitogen-activated protein kinase 1/extracellular signal regulated kinase and nuclear factor kappa-B pathways, limitation of the cyclin-dependent kinase inhibitors (p21 and p27) degradation, prevention of the c-Myc activation, reduction of proinflammatory cytokines, *etc.*, which determine apoptotic responses, tumor-suppressor effects, cell survival reduction, and cell growth inhibition[183,211]. Nevertheless, data from well-designed RCTs (that limit the effect of confounding factors) in support of the HCC chemopreventive effects of statins in NAFLD/NASH population is scant.

For the other anti-hyperglycemic and lipid-lowering drug classes there is no consistent evidence so far regarding potential HCC protective effects.

Resmetirom is a thyroid hormone receptor β-selective agonist, which was shown to significantly reduce the liver fat after 12 and 36 wk of treatment[212]. It is currently under evaluation for safety and efficacy in improving NASH and preventing progression to cirrhosis and/or advanced liver disease in a phase 3 RCT (MAESTRO-NASH; NCT03900429)[213].

Aspirin: Growing evidence coming from preclinical and clinical observational studies suggest that aspirin might play a role in HCC prevention[80,165]. The mechanisms are related to inhibition of selective cyclooxygenase-2 as well as of platelet-derived growth factor, P4HA2, nuclear factor kappa-B activation, and protein kinase 3 signaling, *etc.*, which may prevent proliferation of liver cancer cells and angiogenesis and promote fibrosis resolution[80,165]. Several meta-analyses have explored the potential HCC protective effect of aspirin and have shown that it is associated with reduced incidence/risk of HCC (RRs/HRs/ORs between 0.74 and 0.51)[214-219]. The meta-analysis by Memel *et al*[214] implied stronger association after adjustment for metformin/statin use and accounting for cirrhosis. Also, an inverse relationship seems to exist between aspirin dose/duration of use and HCC risk, but this should be further confirmed[215,219]. There was no evidence of higher risk of bleeding with aspirin use, including in patients with HCC in most meta-analyses except one[216-220]. Moreover, the systematic review and meta-analysis by Tan *et al*[216] showed that aspirin use was associated with improved liver-related mortality (OR: 0.32, 95%CI: 0.15-0.70) and reduced risk of HCC recurrence (HR: 0.80, 95%CI: 0.75-0.86). The same was observed in the meta-analysis by Li *et al*[220], which demonstrated a reduced risk of HCC recurrence (RR: 0.74, 95%CI: 0.59-0.93; $P = 0.01$) and all-cause mortality (RR: 0.59, 95%CI: 0.47-0.73; $P < 0.001$). Although the evidence points toward a potential benefit of aspirin use in prevention of HCC, further prospective data is still necessary in the NAFLD/NASH population.

Vitamin E has anti-oxidative properties, and it might modulate fibrogenesis through inhibition of transforming growth factor β1[12,221]. A recent meta-analysis of eight RCTs reported that vitamin E supplementation (400-800 IU/d, between 8-96 wk) was associated with reduction of fibrosis score (MD *vs* placebo: -0.26, 95%CI: -0.47 to -0.04; $P = 0.02$) as well as a decrease in steatosis, lobular inflammation, and hepatocellular ballooning compared with placebo[222]. However, only one study included patients with T2DM. In fact, the study by Bril *et al*[223] that randomized 105 T2DM patients with biopsy-proven NASH to vitamin E 400 IU b.i.d., vitamin E 400 IU b.i.d. plus pioglitazone 45 mg/d, or placebo did not reach the primary outcome (two-point reduction of NAS from two different parameters, without worsening of fibrosis) in patients receiving vitamin E *vs* placebo but resulted in improvement of steatosis. Thus, it is not clear if the benefit of vitamin E supplementation is restricted to individuals without diabetes, and more data is needed in the T2DM population.

Experimental studies have suggested that antifibrotic therapies may serve as HCC preventive interventions by addressing the underlying cause of carcinogenesis onset[224,225]. Unfortunately, no anti-fibrotic drug has been approved so far by the European Medicines Agency or by the United States Food and Drug Administration for NASH treatment.

Obethicolic acid (OCA) is a farnesoid X receptor agonist shown to improve fibrosis without worsening NASH in an interim analysis of a phase 3 RCT (REGENERATE; NCT02548351)[226,227]. In this analysis, a significantly higher proportion of patients treated with OCA 25 mg daily presented improvement in fibrosis by ≥ 1 stage with no worsening of NASH (23% *vs* 12% in placebo group, $P = 0.0002$)[227]. In the analysis that included patients receiving at least 15 mo of therapy, three times more patients in the OCA 25 mg/d group obtained ≥ 1 stage improvement in fibrosis (38%) *vs* progression of fibrosis (13%) compared with the placebo group, in which similar proportions of patients presented improvement (23%) *vs* worsening (21%) of fibrosis[227]. The FLINT (Farnesoid X Receptor Ligand Obeticholic Acid in NASH Treatment) trial had previously shown that treatment with OCA 25 mg/d for 72 wk improved liver histology (RR: 1.9, 95%CI: 1.3-2.8; $P = 0.0002$) in patients with biopsy-proven NASH[228]. However, in both trials, a higher proportion of patients treated with OCA presented pruritus (23% in FLINT study and 51% in REGENERATE study) in a dose-dependent manner[227,228]. In addition, OCA therapy was associated with increased low-density lipoprotein cholesterol levels, but this could be attenuated by concomitant statin therapy[80,229]. HCC animal model data suggested that the OCA might attenuate the development and progression of NASH-associated HCC by upregulating

sirtuin-1 and modulating the SOCS3/Jak2/STAT3 pathway[230].

Pentoxifylline: Although few small histological studies have suggested liver-related benefits with pentoxifylline 400 mg t.i.d. treatment [*i.e.* improvement of steatosis, lobular inflammation, and liver fibrosis score (mean change: -0.2 *vs* +0.4 on placebo, P = 0.038)] and regression of fibrosis when combined with vitamin E (P = 0.003), data is still scarce and not consistent[231,232]. Four meta-analyses have reported conflicting results with regards to the effect of pentoxifylline on liver fibrosis, with only two of them suggesting benefits[233-236]. Therefore, additional data is required.

Two other initially promising anti-fibrotic medications have failed to prove a significant impact on hepatic fibrosis in phase 3 clinical trials. Elafibranor, an agonist of PPAR-α/δ, reduced steatohepatitis without worsening fibrosis in a phase 2 trial in NASH patients, but the primary end-point was not met [237]. The phase 3 RCT (RESOLVE-IT-NCT02704403) was prematurely discontinued due to limited efficacy at the time of the interim analysis[238]. Cenicriviroc, a dual inhibitor of C–C motif chemokine receptor 2/5, reduced liver fibrosis in NASH patients but did not reach the primary outcome in a phase 2b study[239]. This was followed by a phase 3 trial (AURORA-NCT03028740), which was also terminated early due to lack of efficacy resulting from the planned interim analysis[240]. Several other drugs with potential anti-fibrotic effects are currently in development/evaluation, and the results are expected with interest[241].

CONCLUSION

Regardless of the risk status, all NAFLD/NASH patients should consider adopting lifestyle changes (healthy diet and physical exercise) and controlling their body weight, as these are the cornerstone interventions for NAFLD/NASH management and possibly through altering the natural course of the disease for HCC prevention.

Data suggest a possible role of comprehensive lifestyle changes in reducing the risk of HCC, but specific evidence in NAFLD/NASH patients is rather limited at this point and not sufficient to clearly indicate preventive effects on NAFLD/NASH-associated HCC. Moreover, there is no consensus regarding the composition of a protective diet but decreasing the intake of deleterious nutrients/foods and beverages (*i.e.* saturated fats, sugar-sweetened beverages, alcohol), increasing the beneficial nutrients/foods/beverages (vegetables, coffee, dietary fiber, omega-3 PUFA), and adherence to a healthy dietary pattern (such as the Mediterranean diet or traditional Cantonese dietary pattern) are reasonable and safe approaches. However, their role in HCC prevention still needs to be confirmed by further well-designed prospective studies and experimental research.

Several drug classes (metformin, statins, and aspirin and possibly TZDs, GLP-1 RA, vitamin E, and obeticholic acid) might exert chemopreventive effects by addressing the underlying mechanisms of the disease, but direct evidence regarding their role in NAFLD/NASH-associated HCC prevention is insufficient at the moment.

A timing combination of therapies/non-pharmacological interventions and perhaps adapting them to the stage of disease and/or patient particularities will be necessary to obtain disease resolution and prevention of cirrhosis/NASH-associated HCC.

FOOTNOTES

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Country/Territory of origin: Romania

ORCID number: Simona Cernea 0000-0002-9494-6571; Danusia Onișor 0000-0002-5710-9109.

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REVIEW

Modern drug discovery for inflammatory bowel disease: The role of computational methods

Titilayo Omolara Johnson, Augustina Oduje Akinsanmi, Stephen Adakole Ejembi, Olugbenga Eyitayo Adeyemi, Jane-Rose Oche, Grace Inioluwa Johnson, Abayomi Emmanuel Adegboyega

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Titilayo Omolara Johnson, Augustina Oduje Akinsanmi, Stephen Adakole Ejembi, Olugbenga Eyitayo Adeyemi, Jane-Rose Oche, Abayomi Emmanuel Adegboyega, Department of Biochemistry, University of Jos, Jos 930222, Plateau, Nigeria

Grace Inioluwa Johnson, Faculty of Clinical Sciences, College of Health Sciences, University of Jos, Jos 930222, Plateau, Nigeria

Corresponding author: Titilayo Omolara Johnson, BSc, MSc, PhD, Associate Professor, Department of Biochemistry, University of Jos, Bauchi Road, Jos 930222, Plateau, Nigeria. titijohnson2004@yahoo.com

Abstract

Inflammatory bowel diseases (IBDs) comprising ulcerative colitis, Crohn's disease and microscopic colitis are characterized by chronic inflammation of the gastrointestinal tract. IBD has spread around the world and is becoming more prevalent at an alarming rate in developing countries whose societies have become more westernized. Cell therapy, intestinal microecology, apheresis therapy, exosome therapy and small molecules are emerging therapeutic options for IBD. Currently, it is thought that low-molecular-mass substances with good oral bio-availability and the ability to permeate the cell membrane to regulate the action of elements of the inflammatory signaling pathway are effective therapeutic options for the treatment of IBD. Several small molecule inhibitors are being developed as a promising alternative for IBD therapy. The use of highly efficient and time-saving techniques, such as computational methods, is still a viable option for the development of these small molecule drugs. The computer-aided (*in silico*) discovery approach is one drug development technique that has mostly proven efficacy. Computational approaches when combined with traditional drug development methodology dramatically boost the likelihood of drug discovery in a sustainable and cost-effective manner. This review focuses on the modern drug discovery approaches for the design of novel IBD drugs with an emphasis on the role of computational methods. Some computational approaches to IBD genomic studies, target identification, and virtual screening for the discovery of new drugs and in the repurposing of existing drugs are discussed.

Key Words: Inflammatory bowel disease; Computer-aided drug design; Janus Kinase; Molecular docking; Genome-wide association study; Molecular dynamics simulation

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Core Tip: For the design of small molecule drugs to treat inflammatory bowel disease (IBD), highly effective and time-saving approaches, such as computational methods, are still a viable choice. By complementing experimental studies with computational approaches, the probability of successful drug discovery is increased while simultaneously reducing associated costs. This article provides a summary of the current drug discovery pipeline for IBD, with special emphasis on the part played by computational methods. The use of *in silico* genomic studies, target identification, and virtual screening to find new drugs and repurpose existing drugs for the treatment of IBD are discussed.

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INTRODUCTION

Inflammatory bowel disease (IBD) refers to a group of disorders characterized by chronic inflammation of the gastrointestinal tract that is oftentimes triggered by unknown causes. Crohn's disease (CD) and ulcerative colitis (UC) are the two most prevalent IBDs[1]. The clinical courses are characterized by consistent relapses and recoveries, linked to destructive idiopathic inflammation of the digestive tract. Despite the fact that the precise causes of IBD are still being investigated, it is believed to be the result of an intricate relationship between various parameters, including immune system dysfunction, microbial dysbiosis, a genetic susceptibility, and some environmental factors[2,3].

As the etiology of IBD continues to be a "crossword" to solve, its prevalence and significant global impact continue to rise[4-6]. Globally, the prevalence of IBD increased by 85.1% between 1990 and 2017, and the number of people diagnosed with the disease increased by 95%, as reported by the Global Burden of Disease 2017 Inflammatory Bowel Disease Collaborators. Since 1990, the incidence of IBD has risen in newly industrialized countries in Africa, Asia and South America, including Brazil, to the extent that at the turn of the 21st century this idiopathic disease[7] has become a global disease with accelerating incidence in countries whose societies have become more westernized[8]. Also of interest is the increasing prevalence of microscopic colitis, a subtype of IBD characterized by chronic watery diarrhea caused by colon inflammation. The disease has recently gained more attention due to the availability of more information about its pathogenesis, diagnosis and therapy. The incidence of microscopic colitis is progressively on the rise, approaching that of CD and UC in some populations[9].

Treatments options for IBDs, especially CD and UC, have made significant progress in the last few years[5]. A wide range of anti-inflammatory and symptom-relieving drugs is available for patients with UC. Recent treatments aim to improve the patient's quality of life by alleviating symptoms like abdominal pain and diarrhea and bringing the disease under control as a whole[10]. Conventional treatments include aminosalicylates, corticosteroids, immunomodulators and biologics as part of the pharmacotherapy to control symptoms. When necessary, they also include some general procedures, including surgery. The advent of biologics and other therapeutic advancements in recent years has altered not only the treatment modalities but also the perception of IBD therapy. While symptom remission, complication avoidance and complication treatment are all important, mucosal healing is also a major target. Healing of the mucosa occurs when inflammation in that area subsides and the mucosal lining is returned to its normal structure[11]. Emerging evidence suggests that mucosal healing is linked to improved long-term outcomes, including lower rates of clinical recurrence, hospitalization, surgery, and disability[12,13].

Many patients with IBD have seen a dramatic improvement in their long-term outcomes, both regarding disability and quality of life since the introduction of monoclonal antibodies targeting tumor necrosis factor (TNF) about 20 years ago. However, despite these developments, there are still many unfulfilled needs. For instance, less than a third of treated patients who begin a biologic therapy achieve and maintain drug remission at 1 year. Even in cases when clinical and endoscopic criteria for remission have been met, symptoms such as diarrhea, stomach discomfort, joint problems, and exhaustion may still be experienced[14]. Also, the lack of responsiveness to biologic therapies, which can be caused in part by the protein's immunogenicity upon administration, and the need to discontinue drugs because of intolerance or side effects show that a better generation of therapeutic alternatives is still needed. To achieve the desired results of immune homeostasis restoration and better symptom control, additional progress is still required. Emerging treatment options for IBD include cell therapy, intestinal microe-

cology, apheresis therapy, exosome therapy, and small molecules[15]. Low-molecular-mass compounds with excellent oral bioavailability and the capacity to cross the cell membrane to modulate the functions of parts of the inflammatory signaling pathway are now regarded to be promising therapeutic alternatives for the treatment of IBD. Several small molecule inhibitors are being explored as a possible treatment option for IBD, such as inhibitors of Janus kinase (JAK) enzymes[1]. For the development of these small molecule drugs, the use of highly effective and time-saving techniques, such as computational methods, remain a viable option.

Owing to the intricate nature of the molecular mechanisms involved in disease pathogenesis, the process of developing small molecule drugs is complicated[16,17]. The traditional methods of drug design and development require time-consuming, costly and laborious scientific procedures. On the other hand, computational tools hold great promise as a viable option for the design and development of new small molecules of biomedical relevance[18]. Computer-aided drug design (CADD) employs computer processing power, statistics, mathematics and three-dimensional (3D) graphics to elucidate the affinity and binding mode of small molecules against therapeutic targets.

As a whole, computational methods aid in the identification of candidate target proteins for drug screening and design by searching through large amounts of genomic data for the most important genes [19,20]. In combination with experimental data, protein structures can be predicted with some accuracy [21]. In order to find drug binding sites and understand how drugs work, it is necessary to conduct research into the structural and thermodynamic features of target proteins, both of which can be accomplished with biomolecular simulations using multiscale models[22]. Next, chemical libraries are explored using virtual screening to identify potential drug candidates based on the ligand binding sites on target proteins[23]. With a significantly reduced number of potential drug candidates, *in vitro* and/or *in vivo* experiments can be used to further assess the effectiveness of these molecules. Another CADD approach, besides virtual screening, is provided by *de novo* drug design techniques, which produce highly specific small molecules with good synthetic accessibility[24].

Medicinal chemists use CADD methods to help in rationalizing the selection of hit compounds and in hit-to-lead optimization. CADD has been used as an efficient method for identifying potential lead compounds toward the development of drugs for a wide range of diseases, including IBDs. A variety of computational approaches, such as molecular docking, molecular dynamic (MD) simulation, quantitative structure-activity relationship (QSAR) and pharmacophore modeling, are used to rationally design potent therapeutics with higher efficacies and fewer side effects[25]. The availability of more information about the molecular basis of IBD pathogenesis has further enhanced the use of computational methods in the design of small molecule drugs for IBD treatment. In the pathogenesis of IBD, there is an interplay between various factors such that any stimulus can lead to a cascade of events or even a vicious cycle. This gives a variety of therapeutic targets for which IBD drugs can be designed.

MOLECULAR ASPECTS OF IBD PATHOGENESIS

An interplay of genetic, epithelial and immune factors in the presence of intestinal microbiota are believed to be responsible for the development of the IBD[26].

Genetic factors

The genetic factors causing IBD were initially established through family and twin studies. The disease was observed to cluster in families, with a relative risk of 13-36 for siblings of CD patients and 7-17 for UC patients[27]. Hence, the prevalence of IBD is much higher among first-degree relatives of those with IBD than it is among the general population, suggesting a role for genetic factors in the pathogenesis of the condition. Monozygotic twins exhibit a much higher rate of disease concordance than dizygotic twins, and there are significant differences in the incidence and prevalence of IBD among various populations, according to the twin studies[27]. In CD, the concordance rate for monozygotic twins is approximately 50% compared to 15% for dizygotic twins. Meanwhile, the concordance of monozygotic twins for UC is only 16%, suggesting that genetic factors are less dominant in this form of IBD. In general, there is a growing number of genetic markers linked with the pathogenesis of IBD at various levels including innate immunity, epithelial integrity, antigen presentation, cell adhesion, and drug transporter. Along with environmental and immune system factors, the genetic markers at the different levels play a major role in genetic susceptibility to IBD[28].

Epithelial defects

Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is a cytoplasmic protein that is induced in epithelial cells[29] and is constitutively expressed in neutrophils, macrophages, and dendritic cells[30]. Specifically, the leucine-rich repeat domain of NOD2 is necessary for recognition of muramyl dipeptide, a fragment of peptidoglycan present in bacterial cell walls. Ultimately, muramyl dipeptide causes nuclear factor kappa B activation and the induction of proinflammatory cytokines[31, 32]. Activation of the innate and adaptive mucosal immune responses as a result of NOD2 polymorphism causes the synthesis of cytokines, metalloproteinases and enzymes, which subsequently

results in tissue destruction and epithelial barrier defect.

Evidence from genome-wide association studies (GWAS)[33,34] also shows the involvement of hepatocyte nuclear factor 4α (HNF4α) in the pathogenesis of UC. HNF4α is a member of the nuclear receptor superfamily of ligand-dependent transcription factors that is highly conserved and well expressed in liver and gastrointestinal organs. Several studies have linked HNF4α to intestinal epithelial differentiation, lipid metabolism and epithelial junctions[27,35], which are important components in colon development[36]. A zebrafish model suggests that the gut microbiota negatively regulates expression levels of HNF4α[37]. There is evidence linking alterations in HNF4 expression and activity, as well as germline variants, to IBD and colorectal cancer[38,39]. In UC, polymorphisms of HNF4α result in defects of epithelial tight junctions and intestinal permeability. Due to these epithelial barrier defects, there is transepithelial influx of bacteria, activation of the immune responses, release of cytokines, and further increase in epithelial junction permeability[40-42].

The development of UC has also been linked to a decrease in fatty acid oxidation in the colonic mucosal epithelium. Carnitine is a necessary cofactor in lipid metabolism and may be transported by the organic cation transporter (OCTN), a family of transporter proteins for organic cations. Carnitine helps shuttle long-chain fats into the mitochondria. Both OCTN1 and OCTN2 have been linked to CD-causing mutations: SLC22A4 1672C/T for OCTN1 and SLC22A5 +207G/C for OCTN2. The TC haplotype, formed by the presence of one or more of these mutations, is linked to the development of ileal, colonic and perineal symptoms and the requirement for surgical treatment of CD[28,43].

A number of tissues including the colon, small intestine, placenta, liver, heart and skeletal muscle contain high levels of Drosophila long disc homologue 5. The Drosophila long disc homologue 5 gene belongs to the membrane-associated guanylate kinase gene family, which encodes cell scaffolding proteins. It is thought to be involved in the maintenance of epithelial cell integrity and in signal transduction. Mutations in this gene have been linked to an increase in intestinal permeability[28]. The D haplotypes were found to be associated with UC and CD in a European cohort[44]. CD patients in Japan also showed another form of this gene (rs37462)[45]. Patients with IBD were found to have an excess transmission of haplotype D, which is defined by the haplotype-tagging single nucleotide polymorphisms (SNPs) G113A[28].

Mucosal immune responses

In normal intestinal mucosal immune response, the presence of gut microbiota has conditioned the dendritic cells present in the epithelium so that there is activation of T regulator cells that produce anti-inflammatory cytokines interleukin (IL)-10 and transforming growth factor-beta (TGF-β) as well as suppression of T effector cells. However, in IBD, the dendritic cells respond to gut microbiota by activating CD4+ T effector cells that differentiate into T helper (Th1, Th2 and Th17) cells depending on the IBD type[40,42]. Recently, the proinflammatory cytokine IL-12 family, which includes IL-22, IL-23, IL-25, and IL-27, has been linked to the pathophysiology of CD and other immune-mediated disorders [46]. The IL-12 family is responsible for the differentiation of Th cells into Th1 cells. IL-12 and IL-23 are heterodimeric proteins with a unique subunit linked to a shared p40 subunit. Patients with CD have elevated levels of these subunits[47,48]. Ustekinumab, a Food and Drug Administration (FDA)-approved drug for moderate-severe CD, inhibits IL-12 and IL-23 receptors on T cells, natural killer cells, and antigen-presenting cells[49]. In addition, several monoclonal antibodies have been discovered that target the Th1/Th17 pathway *via* IL-23. However, none of them have progressed to phase 3 trials[50,51].

The upregulation of IL-13, another crucial cytokine in the Th2 immune response, is considered to be a major trigger of mucosal inflammation in UC patients[52,53]. Research in mice revealed an increase in IL-13 in colitis, and this increase could be mitigated by inhibiting the ability of IL-13 to bind to its signaling receptor[54]. Tralokinumab is a human immunoglobulin G4 monoclonal antibody that binds to IL-13 and inhibits its activity.

Molecular studies have also identified NOD2 as a susceptibility gene in CD. The gene encodes a protein that transduces signals to activate nuclear factor kappa B in monocytes and functions as an intracellular receptor for bacterial products. Muramyl dipeptide activates NOD2, leading to autophagy in dendritic cells. Defective autophagy induction and decreased bacterial localisation in autophagolysosomes are observed in CD patients with susceptibility polymorphisms in the NOD2 gene. Genetic analyses have identified two other genes that are involved in autophagy and intracellular bacteria clearance, namely *IRGM* and *ATG16L1*, demonstrating the importance of autophagy in IBD immune responses. Other genes related with autoimmune disease, such as IL23R and PTPN2, reveal an additional feature of CD pathophysiology[38,40].

Another major player in IBD pathogenesis is TGF-β1, an essential factor in the healing of intestinal cells and the reduction of inflammation[55]. It is an immunosuppressive cytokine with the ability to inhibit pathogenic T cell activity and antigen-presenting cell responses. Increased levels of Mothers against decapentaplegic homolog 7 (SMAD7), an intracellular protein that binds TGF-β1 receptor and prevents TGF-β1- and SMAD-associated signaling, are believed to account for the deficiency in TGF-β1 activity observed in IBD patients. Mongersen, an oral medication that inhibits SMAD7 activity after administration to the terminal ileum and right colon is being investigated for efficacy in CD[56]. In its current form, the site of action is limited to the terminal ileum and right colon, hence it may not be beneficial for patients with more complicated CD or post-operative recurrence[57].

The involvement of the JAK family of intracellular non-receptor tyrosine protein kinases in the pathogenesis of a number of autoimmune diseases has also been demonstrated[58]. Many autoimmune diseases, including IBD, have been linked to specific SNPs in the genome, and the, JAK/signal transducer and activator of transcription proteins (JAK/STAT) signaling pathway has yielded several cytokines and receptors as potential treatment targets[59]. The JAK/STAT pathway interferes with a couple of inflammatory pathways and development of disease as seen in CD and UC patients is characterized by imbalanced effector T cells, leading to increased effector Th cells (Th1, Th2 and Th17 cells), thus mediating the inflammation[60]. Figure 1 shows a schematic description of the JAK/STAT pathway leading to the expression of genes encoding some inflammatory markers. Inhibition of JAK leads to the inhibition of signaling of a specific subset of cytokines, which are implicated in inflammation[61]. Some small molecule inhibitors of the JAK/STAT pathway with good oral bioavailability have been developed. A very good example is tofacitinib. When compared to monoclonal antibodies, JAK inhibitors have the therapeutic advantage of being able to target multiple downstream signaling pathways induced by inflammatory cytokines, whereas monoclonal antibodies can only block a single molecule[62].

The successful development of tofacitinib, as well as the promising results of other JAK inhibitors in both UC and CD (as shown in Figure 2), demonstrate that JAK inhibition has a role in the treatment of IBD. However, long-term safety studies in people with rheumatoid arthritis and UC who took tofacitinib showed a higher risk of reactivation of herpes zoster, especially at higher doses[63]. This increased risk is likely a class effect of JAK inhibitors and related to IFN and IL-15 inhibition. Also, there might be a possible thrombogenic risk, as shown by patients with rheumatoid arthritis[64]. More selective JAK-1, JAK-3 or tyrosine kinase 2 inhibitors are expected to improve safety while maintaining efficacy. However, these drugs are still systemic; a gut-selective JAK inhibitor with high intestinal exposure and target engagement but no systemic effects are still needed for treating people with IBD. Continued progress is being made in all of these areas[65].

IBD DRUG DISCOVERY AND DEVELOPMENT

The process of identifying a therapeutic agent for extensive study as a potential drug candidate is known as drug discovery[66]. In general, the modern drug discovery process involves identifying a condition to be treated and its unfulfilled medical necessity, selecting a druggable molecular target and validating it, developing *in vitro* assays followed by high-throughput screening of compound libraries against the target to identify hits and optimizing hits to create lead compounds with acceptable potency and selectivity towards the biological target and demonstrated efficacy in an animal model. Following that, the lead compounds are further refined to improve their effectiveness and pharmacokinetics before moving further with drug development (Figure 3). The discovery and development of innovative drugs is a complex process that takes around 12 years and an average of $1.8 billion to bring a new medication to market[66,67].

The process of IBD drug discovery begins with target discovery and selection followed by biological confirmation in cellular and animal models[68]; this is usually the pre-clinical phase. Several therapeutic targets of IBD have been identified, and some are listed in Table 1. Target identification in IBDs is based on qualitative relevance to the pathogenesis of the disease as well as increased magnitude of expression [68]. Biochemical pathways and specific proteins are targeted, and the drugs are developed based on the understanding of the mechanism of action of these targets. This is followed by pharmaceutical developments such as screening for safety, toxicity, pharmacokinetics and efficacy. At the preclinical stage, drugs are tested with targets to investigate levels of interactions and outcomes. Models and methods used differ between laboratories based on specific targets of interest.

For instance, in IBD, targets such as JAK/STAT and inflammatory mediator interleukins such as IL-12, IL-23, IL-6, IL-22Fc[69], tyrosine kinase and toll-like receptors[70] are explored using biologic agents at different stages of development. Following the *in vivo* studies using murine and human T cells, JAK/STAT inhibition by tofacitinib[71] has led to further application of the drug in several clinical trials. Integrins, a class of receptors that facilitate T lymphocyte trafficking into the gut[72] are targets as well. Integrins facilitate adhesive interactions between lymphocytes and endothelial cells, which leads to the trafficking[73] and can lead to T cell-dependent chronic intestinal inflammation in IBD[70]. Etrolizumab, an anti-adhesion agent approved by the FDA for treatment of IBD was subjected to *in vitro* testing, where it internalized β_7 integrin using cell culture models[74]. Etrolizumab selectively inhibits α 4β7 and αEβ7, which are involved in T lymphocyte homing in the gut[75]. Although there are challenges involved in the use of *in vivo* and *in vitro* methods in establishing IBD[68], conditions for the studies of potential drugs are optimized. The molecules with desired preclinical effect on the target go into clinical trial.

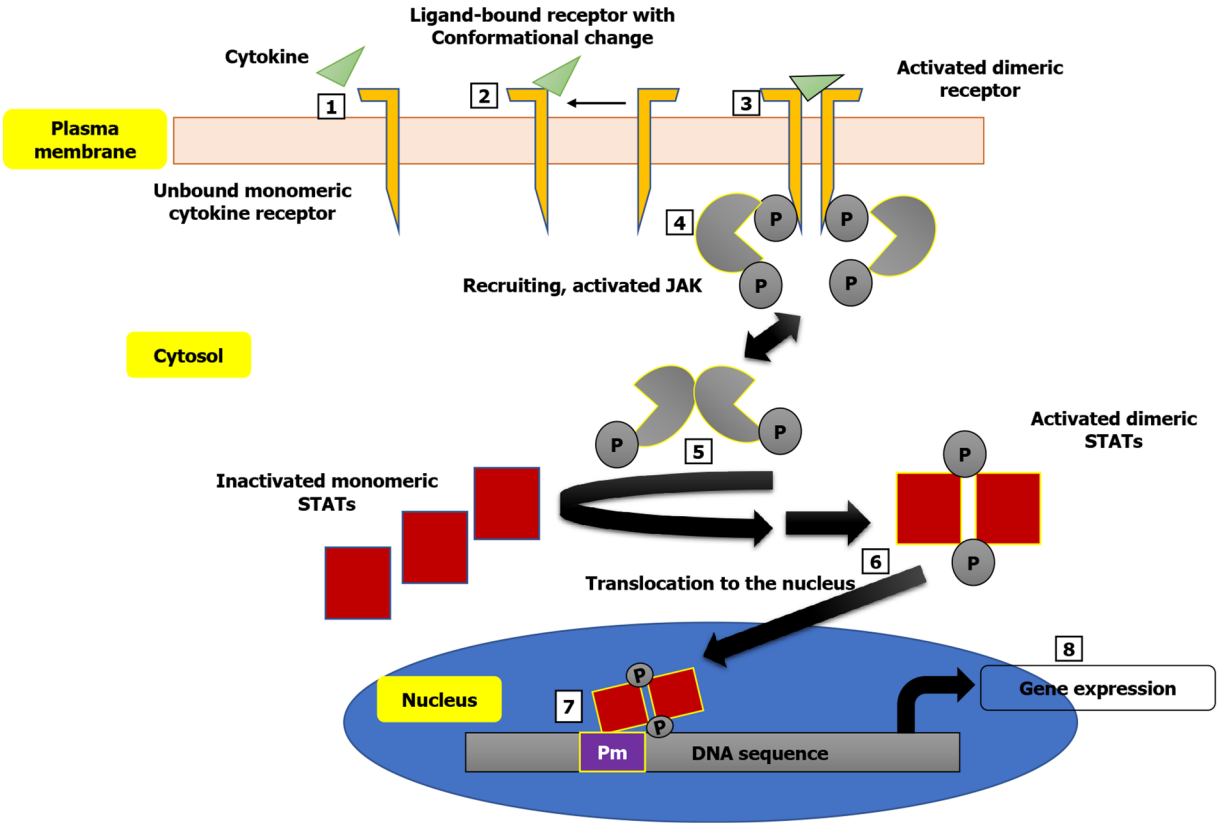
In clinical trials, drugs that scale through preclinical studies are expected to maintain remission in long-term mucosal healing. Clinical trial phases are regarded as long and extensive periods that take years from the point of research to the stage of approval of a candidate drug. Prior to these trials, detailed protocols that show the characteristics of the studies are provided[76]. The clinical phase comes

Table 1 Molecular targets for inflammatory bowel disease drug discovery

Target name	Abbreviation	Description	Disease implication	Modulatory effect of drug
Integrin alpha-4	ITGA4	A member of the family of integrins. Integrins alpha-4/beta-1 (VLA-4) and alpha-4/beta-7 are fibronectin and VCAM1 receptors. Integrin alpha-4/beta-7 is also a MADCAM1 receptor. On activated endothelial cells, VLA-4 integrin induces homotypic aggregation in the majority of VLA-4-positive leukocyte cell lines. ITGA4: ITGB1 binds fractalkine (CX3CL1) and may function as its coreceptor in fractalkine signaling dependent on CX3CR1[123]	ITGA4 upregulated in irritable bowel disease (IBD)	Inhibition
Interleukin 12B	IL12B	IL12B is also known as natural killer cell stimulatory factor 2 or p40, and it associates with IL23A to form IL23, a known stimulator of the JAK/signal transducer and activator of transcription (STAT) signaling pathway and a pathway with proven importance in IBD[124]	IL12B upregulated in IBD	Inhibition
Tumor necrosis factor	TNF	A type of cytokine, which binds to TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. It is secreted by macrophages and is capable of triggering cell death of most tumor cell lines, although capable of promoting cell proliferation and induce cell differentiation under certain conditions[123]	TNF upregulated in IBD	Inhibition
Janus kinase 2	JAK2	A class of kinase, a non-receptor kinase that phosphorylates specific tyrosine residues on the cytoplasmic tails of the receptor. In the cytoplasm, JAK2 plays a pivotal role in signal transduction *via* its association with type I receptors such as growth hormone (GHR), prolactin (PRLR), leptin (LEPR), erythropoietin (EPOR), thrombopoietin (THPO) or type II receptors including IFN-alpha, IFN-beta, IFN-gamma, and multiple interleukins. It stimulates cell growth, development, differentiation or histone modification[123]	JAK2 upregulated in IBD	Inhibition
Prostaglandin-endoperoxide synthase 1 and 2	PTGS1/2	Also referred to as cyclooxygenase; are the primary enzymes involved in the synthesis of prostaglandin. They act both as a dioxygenase and as peroxidase, having two isozymes PTGS1 and PTGS2. This gene encodes the PTGS2 inducible isozyme. Its involvement in prostanoid-dependent inflammation and mitogenesis can be related to their regulation by specific stimulation[123]	PTGS1/2 upregulated in IBD	Inhibition
Peroxisome proliferator activated receptor gamma	PPARγ	A nuclear receptor. It consists of a group of approximately 50 transcription factors involved in many biological processes. It controls some regulatory genes involved in lipid metabolism and insulin sensitization as well as in inflammation and cell proliferation. It is highly expressed in the colon and majorly involved in bacterial-induced inflammation, also mediating the common aminosalicylate activities in IBD[125]. It acts as a critical regulator of gut homeostasis by suppressing nuclear factor-kappa B-mediated proinflammatory responses	PPARγ downregulated in IBD, mostly ulcerative colitis	Activation
Integrin subunit beta 7	ITGB7	Integrin alpha-4/beta-7 is an adhesion molecule that mediates lymphocyte migration and homing to gut-associated lymphoid tissue (GALT). The vascular endothelium of the gastrointestinal tract expresses MADCAM1, an adhesion molecule, which is the medium integrin alpha-4/beta-7 interacts with the gastrointestinal tract. VCAM1 and fibronectin found on the extracellular matrix of the cell also interacts with the integrin. Interaction with fibronectin is due to the CS-1 region[123]	ITGB7 upregulated in IBD	Inhibition
Nuclear receptor subfamily 3 group C member 1	NR3C1	This is a receptor recognized by glucocorticoids. It modulates the activities of cortisol and acts as a transcription factor that modulates the expression of its target genes[126]. It modulates inflammatory responses, cellular proliferation and differentiation in target tissues	NR3C1 downregulated in IBD	Activation
Janus kinase 3	JAK3	Non-receptor tyrosine kinase involved in signal transduction in the cytoplasm *via* its association with type I receptors sharing the common subunit gamma such as IL2R, IL4R, IL7R, IL9R, IL15R, and IL21R. It also plays a vital role in STAT5 activation. IBD pathology is associated with receptor-mediated signaling through the JAK and STAT DNA-binding families of proteins[127]	JAK3 upregulated in IBD	Inhibition
Arachidonate 5-lipoxygenase	ALOX5	ALOX5, an important member of the lipoxygenase gene family, exclusively involved in IBD development[128].	ALOX5 upregulated in IBD, especially	Inhibition

		Catalyzes the oxygenation of arachidonate (5Z,8Z,11Z,14Z)- eicosatetraenoate) to 5-hydroper-oxyeicosatetraenoate (5-HPETE) followed by the dehydration to 5,6- epoxyeicosatetraenoate (leukotriene A4/LTA4), the steps in the biosynthesis of leukotrienes, that mediates inflammation[123]	ulcerative colitis	
Tyrosine kinase 2	TYK2	A non-receptor kinase that carries out both structural and catalytic roles in numerous cytokines and interferons signaling. TYK2 plays a key role in mediating signaling and functional responses downstream of the IL-12, IL-23, and type I interferon (IFN) receptors and TYK2-mediated IL-12, IL-23 and type I IFN signaling activates STAT-dependent transcription, which promotes chronic inflam-mation[129]	TYK2 upregulated in IBD	Inhibition
Phosphoribosyl pyrophosphate aminotransferase	PPAT	PPAT is a key rate-limiting reaction in purine biosynthesis, transferring gamma-nitrogen from glutamine to 5-phosphoribosyl pyrophosphate (PRPP)[130]	PPAT upregulated in IBD	Inhibition
Vitamin D receptor	VDR	A nuclear, ligand-dependent transcription factor that regulates the expression of T cells and genes involved in different physiological functions when in complex with hormonally active vitamin D, 1,25(OH)2D3[131]. VDR plays a multifaceted role in the pathogenesis of IBD and is crucial in regulating autophagy, immune response, tight junctions, gut microbiome, and metabolites[132]	VDR downregulated in Crohn's disease	Activation
Matrix metallopeptidase 1	MMP1	An interstitial collagenase, that digests the spiral structure of collagen types I, II, III and X, subjecting them to hydrolysis by gelatinase and are major players in extracellular matrix degradation[123]	MMP1 upregulated in IBD	Inhibition
Matrix metallopeptidase 7	MMP7	A metallopeptidase member necessary for neutrophil migration into the airspaces by cleaving syndecan-1, a heparin sulfate proteoglycan necessary for the establishment of a neutrophil chemokine gradient[133]. Degrades casein, gelatins I, III, IV and V, and fibronectin and is responsible for the activation of procollagenase[123]	MMP7 upregulated in IBD	Inhibition
Dihydrofolate reductase	DHFR	An enzyme that converts dihydrofolate to tetrahydrofolate in folate metabolism and involved in purine and mitochondrial thymidylate biosynthesis[123]	DHFR upregulated in Crohn's disease	Inhibition
Matrix metallopeptidase 13	MMP13	A member of the family of collagenases. Matrix substrates of MMP13 include native collagen, gelatin and aggrecan. Lipopolysaccharide (LPS)-induced shock and dioctyl sodium sulfosuccinate (DSS)-induced colitis induce MMP13 upregulation in the gut, which results in MMP13-mediated shedding of transmembrane-bound TNF and release of bioactive, soluble TNF, thus triggering a cascade that leads to leakage of intestinal components, which increases systemic inflammation and subsequent organ damage[134]	MMP13 upregulated in IBD	Inhibition
Sphingosine-1-phosphate receptor 1	S1PR1	A type of G-protein-coupled receptor. S1P binds to the S1PR1, which triggers angiogenesis, endothelial barrier enhancement, blood vessel constriction, heart rate modulation and lymphocyte trafficking[135]	S1PR1 downregulated in IBD	Activation
ATPase H+/K+ transporting subunit alpha	ATP4A	A P-type cation-transporting ATPase. The gastric H+, K+-ATPase is a heterodimer made of high molecular, weight catalytic alpha subunit with a glycosylated beta subunit. It is a proton pump that catalyzes the hydrolysis of ATP coupled with the exchange of H (+) for K (+) ions across the plasma membrane and also responsible for gastric acid secretion due to its ability to generate proton gradient across the membrane[123]	ATP4A upregulated in IBD	Inhibition

IBD: Inflammatory Bowel Disease; ITGA4: Integrin alpha-4; IL: Interleukin; TNF: Tumor necrosis factor; JAK: Janus kinase; PTGS1/2: Prostaglandin-endoperoxide synthase 1 and 2; PPARγ: Peroxisome proliferator activated receptor gamma; ITGB7: Integrin subunit beta 7; NR3C1: Nuclear receptor subfamily 3 group C member; ALOX5: Arachidonate 5-lipoxygenase; TYK2: Tyrosine kinase 2; PPAT: Phosphoribosyl pyrophosphate aminotransferase; VDR: Vitamin D receptor; MMP: Matrix metallopeptidase; DHFR: Dihydrofolate reductase; S1PR1: Sphingosine-1-phosphate receptor 1; ATP4A: ATPase H+/K+ transporting subunit alpha; VLA-4: Very late antigen-4; VCAM 1: Vascular cell adhesion molecule 1; MADCAM1: Mucosal vascular addressin cell adhesion molecule 1; CX3CL1: C-X3-C Motif Chemokine Ligand 1; CX3CR1: C-X3-C Motif Chemokine Receptor 1; STAT: Signal transducer and activator of transcription; TNFRSF: Tumor necrosis factor receptor superfamily; GHR: Growth hormone receptor; PRLR: Prolactin receptor; LEPR: Leptin receptor, EPOR: Erythropoietin receptor; THPO: Thrombopoietin; IFN: Interferon; PTGS1&2: Prostaglandin G/H synthase 1 & 2; GALT: Gut-associated lymphoid tissue; 5-HPETE: 5-hydroperoxyeicosatetraenoate; LTA4: Leukotriene A4; prpp: Phosphoribosyl pyrophosphate.



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Figure 1 A schematic description of the Janus kinase/STAT pathway. 1: Cytokine and cytokine receptor before interaction; 2: Ligand-reception interaction brings about conformational change that recruits the second cytokine receptor to dimerize with the first cytokine receptor; 3: The activated dimeric receptor recruits Janus kinase (JAK); 4: Thereby causing JAK to phosphorylate itself and the cytoplasmic tail of the cytokine receptor on the tyrosine residue; 5: Activated JAK phosphorylates monomeric STAT transcription factor, hence causing it to disengage from its endogenous inhibitor and dimerize; 6: The activated dimeric signal transducer and activator of transcription translocates to the nucleus; 7: Where it binds to a promoter; 8: That causes the gene expression of some inflammatory markers. JAK: Janus kinase; STAT: Signal transducer and activator of transcription.

after certainty has been established that the drugs/molecules are effective and non-toxic. Clinical trials involve three main phases before approval, known as phase I, phase II, and phase III. The first two phases are early phases.

The phase I is the stage in drug development when testing and pharmacokinetics studies are carried out using healthy or ill subjects[76] to ascertain safety level of the drugs. The pharmacokinetics and pharmacodynamics of the drugs are determined using volunteer patients or patients who are not responsive to previous treatments in order to determine drug safety alongside desired clinical activities and an Absorption, Digestion, Metabolism and Excretion (ADME) profile[68]. For instance, a phase I study of Japanese patients with UC treated with vedolizumab, an anti-integrin antibody, showed an appreciable level of tolerance in the desired clinical activity of the drug as well as a satisfactory level of pharmacodynamics and pharmacokinetics[77]. The outcome of this phase determines whether the drug scales through to the next phase. The phase I studies can last for up to many years and part of the process involves identification of adverse effects or events other than the occurrence(s) observed in the preclinical stage. Foreknowledge of a possible mechanism of action of a drug can also guide expectations in clinical trials.

The phase II is the stage that involves dose ranging. The efficacy of the drugs are determined alongside the optimum dose that would be considered safe[76]. At the point of dose determination, the outcome of the effect of different doses would guide the decision to proceed to the next phase. A phase II study of varied doses of ozanimod, an agent that targets sphingosine-1-phosphate receptors 1 and 5, was used to treat adult patients with moderate to severe CD based on an uncontrolled multicenter trial within 12 wk and the effects of different doses at different intervals were observed[78]. A drug that scales through is launched into the third phase.

Phase III is the stage whereby the efficacy of the drug is compared with that of an already established standard. Patients recruited for this phase are usually from the general population compared to phase I and II. The side effects of the drugs are studied as well as their effectiveness, comparing them with common treatments to help guide safety and proper use. Currently, etrolizumab, is undergoing a phase III clinical trial for treatment of IBD using six multicenter prospective randomized controlled trials and two open-label extension studies[75]. Upon conclusion of the studies, the drugs are considered for

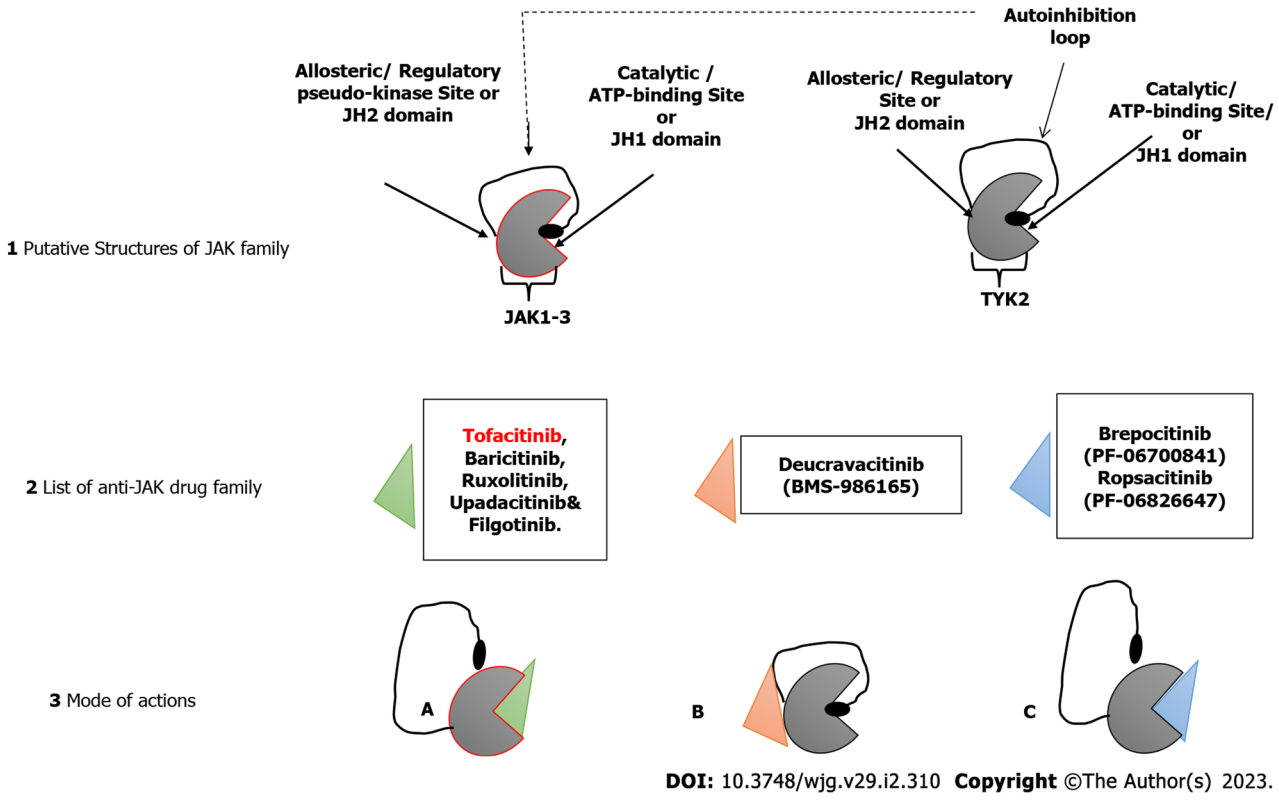


Figure 2 Putative representations. 1: Janus kinase (JAK) 1, 2 or 3, and tyrosine kinase 2 (TYK2) with their respective catalytic/ATP-binding/Janus homology (JH) 1 site, regulatory/allosteric/JH2 site and autoinhibition loop; 2: List of anti-JAK drug family differentiated with green, pink and blue shapes; 3: Different modes of action of the drugs differentiated with the colors of the shape: (A) Drugs in group A (*e.g.*, tofacitinib, baricitinib, ruxolitinib, upadacitinib and filgotinib) block the ATP-binding site of either/both JAK 1 or 2, while the inhibition loop is disengaged; (B) Drugs in group B (*e.g.*, deucravacitinib) bind to the allosteric site of TYK2, thereby stabilizing the autoinhibition loop from disengaging from the catalytic domain; (C) Drugs in group C (*e.g.*, brepocitinib and ropsacitinib) are dual inhibitors and have a similar mode of action as "A" by binding to the catalytic site of their targets but blocking the catalytic site of both JAK 1 and TYK2 or JAK2 and TYK2, respectively. JAK: Janus kinase; TYK: Tyrosine kinase; JH: Janus homology.

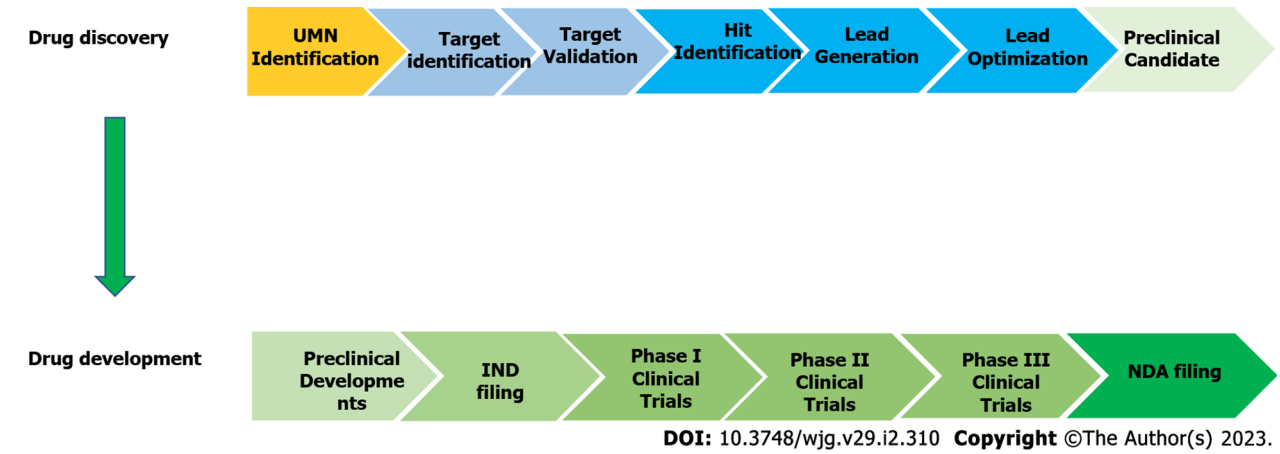


Figure 3 Overview of the drug discovery and development process. IND: Investigated new drug application; NDA: New drug application; UMN: Unmet medical needs.

approval based on FDA standards. Drugs are reviewed by the FDA based on the evidence of preclinical and clinical outcomes and approved for marketing based on the terms of the FDA.

ROLE OF COMPUTATIONAL METHODS IN IBD DRUG DISCOVERY

The use of computational methods has become a crucial part of the drug discovery process[18]. A

number of commercially available medications as well as several clinical candidates have been discovered or improved with the help of molecular modeling techniques. CADD combines multiple chemical-molecular and quantum approaches to the discovery, design and development of drugs. Structure-activity relationships form the basis of many CADD methods. The objectives of CADD are multidisciplinary in nature, with the end goal being the modification of bioactive molecules, the formation of therapeutic alternatives and the knowledge of biological events at the molecular level[79].

From drug identification to pharmacological role discovery and from preclinical testing to drug marketing, computers have played a fundamental role in the entire drug discovery and development process[15]. In addition, CADD opens the door to the possibility of drug repurposing, which is the systematic identification of new potential uses from drugs that have already been approved for other indications[79]. According to the results of a computational drug repurposing study conducted by Bai *et al*[80], atorvastatin shows promise as a novel treatment approach for improving symptoms in patients with UC. They developed a framework for systematically integrating publicly accessible heterogeneous molecular data with clinical data on a large scale in order to repurpose FDA-approved drugs for a wide variety of human diseases.

Computational methods are useful in various aspects of modern drug discovery for IBD. These include but are not limited to genetic studies for the identification of pathways linked to IBD pathogenesis, target identification, and virtual screening.

IBD genetic studies

Computational methods have made tremendous contributions to the field of IBD genetic studies. As mentioned earlier, genetic factors contribute to the pathogenesis of IBD and the understanding of the molecular events are important for target identification. The advent of GWAS is a turning point in the history of genetic research into complex human disease. GWAS is a technique for identifying genetic markers that are linked to an increased probability of developing a disease or exhibiting a certain trait. The strategy entails analyzing the genomes of a large number of people to identify genetic variations that are more common in people who have a disease or a particular trait than in those who do not. Following the discovery of such genomic variants, researchers often use GWAS to search for other potentially causal variants located in close proximity[81]. IBD is a prime example of the usefulness of GWAS and related analyses. Using GWAS based on SNPs, researchers were able to identify 163 genetic loci and numerous signaling pathways that are linked to IBD[82]. Many of these loci have pleiotropic effects, and risk prediction models were developed using a wide variety of genetic variations. Key contributions of the gut microbiome and important interactions between genes and the environment are emerging as a result of these studies[83].

The development of computational methods has contributed significantly to the identification of the vast quantity of genetic determinants. There are a number of different software packages available for use in GWAS research[84], and a common example is PLINK. In addition to standard GWAS features like quality control filtering and SNP association testing, PLINK also includes more advanced tools like gene-based analysis, annotation and epistasis testing in a compact, user-friendly package. PLINK initially discovered the actin-related protein 2/3 complex subunit 2[85], IL10, HNF4a, cadherin-3, cadherin-1 and laminin subunit beta-1[33] loci and recently discovered 163 IBD loci[82]. In the majority of GWAS-based genetic studies of IBD, PLINK has served as the primary analytical tool. A linear mixed model, which accounts for both fixed and random effects, has been used in GWAS in recent years. Factored Spectrally Transformed Linear Mixed Models[86], Efficient Mixed-Model Association expedited[87] and Genome-wide efficient mixed-model association (GEMMA)[88] are all examples of linear mixed model-based software. They are able to adjust for both overt and covert connections within a population simultaneously. Multiple correlated phenotypes can be analyzed with GEMMA as well.

There is a plethora of pathway analysis software packages available, many of which are based on GWAS[89]. The development and implementation of GWAS-based pathway software has frequently been illustrated with CD as an example. To examine the CD dataset from the Wellcome Trust Case Control Consortium, Wang *et al*[90] used the GenGen software to find significant associations of the IL12/IL23 pathway with CD status in multiple cohorts genotyped with different SNP chips and from different ethnic backgrounds[91]. This was among the first analyses of IBD pathways based on GWAS data. IL3 activation and signaling pathway were also linked to CD, based on the research by Torkamani *et al*[92]. They used the MetaCore program, which is a for-profit product created by GeneGo Inc. (St. Joseph, MI, United States).

Using the statistical technique of Simes/false discovery rate, Peng *et al*[93] discovered significant enrichment of the JAK/STAT signaling pathway, as well as the cytokine-cytokine receptor interaction. With a model-based strategy, Carbonetto *et al*[94] found a similar result for a number of cytokine signaling pathways. Holmans *et al*[95] used the software ALIGATOR to discover the involvement of MHC genes in addition to IL/cytokine signaling. Similarly, Jostins *et al*[82] examined the largest IBD GWAS to date for enrichment of canonical pathways or Gene Ontology terms and discovered significant enrichment of Gene Ontology terms pertaining to the regulation of cytokine production, lymphocyte activation, and the JAK/STAT signaling pathway.

It is possible that the differences between the studies can be attributed to the different statistical methods and/or the different pathway databases used in their studies. However, all of these pathways were related to interleukins and the immune system. Torkamani *et al*[92] found a significant association between calcium signaling and the carbohydrate response element binding protein regulation pathway; Peng *et al*[93] found a significant association between ABC transporters and the extracellular matrix receptor interaction. Numerous user-friendly web-based software programs, such as improved Gene Set Enrichment Analysis for (i-GSEA4) GWAS[96] and GSEA-SNP[97], have been developed for pathway analysis using GWAS data.

IBD target identification

Following the discovery of the biological basis of a disease, the next step in drug discovery is the identification of potential drug targets. The most promising targets for drug discovery are hypothesized to be highly prevalent in the disease-affected population, to have a well-established function in the underlying pathology and to be directly linked to the disease of interest. Potential drug targets are defined as disease-modifying rather than disease-causing. In the present day, a wide range of methods, both experimental and computational, are used to identify drug targets. The experimental methods rely heavily on comparative genomics, with phenotype and gene association analysis serving as complementary tools. All experimental methods yield credible findings, but they have significant drawbacks, including the high cost and extensive scientific labor needed to experimentally probe the entire space of chemical compounds to identify viable drug targets[98]. In light of these drawbacks, researchers and pharmaceutical companies increasingly rely on computational methods for initial investigations before turning to experimental approaches for validation and other purposes.

Various bioinformatics resources are available for the identification of drug targets as illustrated in Table 2. These programs efficiently process a large volume of data from genomic, transcriptomic and proteomic databases and ultimately provide potential drug targets in a short period of time and at a low cost. Several computational methods are currently accessible, each of which makes use of a unique type of molecular information, such as a gene or genomic sequence, molecular interaction data or the 3D structure of a protein[98]. There are strong connections between most of these methods.

Mohan *et al*[99] reported using genetic databases to find new molecular targets for IBD. They used four different genetic databases to categorize the protein-coding genes associated with UC (3783 genes), CD (3980 genes), uveitis (1043 genes), arthritis (5583 genes), primary sclerosing cholangitis (1313 genes), and pyoderma gangrenosum (119 genes). The databases used were Genecards: The Human Gene Database, DisGeNET, the Comparative Toxicogenomics Database, and the Universal Protein Resource. Then, they used Network Data Exchange to map a distinct signal pathway based on the identified common genes underlying the aforementioned diseases. Across UC, CD, uveitis, arthritis, pyoderma gangrenosum and primary sclerosing cholangitis, they identified a distinct set of 20 genes with the highest probability of overlap. Different disease processes were linked to some unique immune modulators. IL-25 and monensin-resistant homolog 2 were observed in UC, CD, pyoderma gangrenosum, and arthritis. Arachidonate 5-lipoxygenase was found to contribute to the development of UC, CD, and arthritis. The involvement of solute carrier organic anion transporter family member 1B3 is unique to pyoderma gangrenosum, UC, and CD. TNF was found to be involved in the pathogenesis of UC, CD, psoriatic spondylitis, and arthritis.

Virtual screening

Drug discovery relies heavily on the physical screening of large chemical libraries for biological targets in order to find new lead compounds. High-throughput screening is a method for finding active molecules in experiments by analyzing more than a million compounds biochemically. However, developing and deploying this technology takes a long time and a sizable investment. Therefore, virtual high-throughput screening was developed as a more affordable and effective calculation method. This technique has seen extensive use in the earliest stages of drug discovery. The goal is to search through huge compound libraries to find the structure of a novel, active small molecule. To some extent, this supports the goals of high-throughput screening. Virtual screening saves money by reducing the number of compounds used to measure pharmacological activity, while high-throughput screening uses all compounds in the database[18,24,100]. In this sub-section, we will go over the various virtual screening techniques that are commonly used in IBD drug discovery.

Molecular docking: Molecular docking is commonly used in the drug discovery and development process because of its ability to predict interaction patterns between proteins and small molecules as well as proteins[18,101,102]. The principle behind this phenomenon proposes that ligand and receptor recognition is predicated on a similarity in spatial shape and energy. Understanding the action mechanism of a drug requires first establishing its binding conformation to a specific protein receptor [24]. The goal of docking is to accurately assess the strength of binding by fitting the structure of a ligand within the requirements of a receptor binding site[103]. The most frequently cited molecular docking software packages for use in drug discovery are summarized in Table 3.

Table 2 Bioinformatics resources for the identification of drug targets

Tool/Database	Description
Open Targets Platform	To facilitate systematic target identification and prioritization for drug discovery based on underlying evidence, the Open Targets Platform offers users a searchable knowledgebase and user interface[123]
SELF-BLM	A self-training support vector machine-based bipartite local model that predicts drug-target interactions[136]
iDTIESBoost	A model for detecting drug-target interactions based on evolutionary and structural features[137]
GEO	Database that stores array- and sequence-based transcriptomics data that can be applied to functional genomics[138]
DASPfind	Predicts drug-target protein interactions that stem from shared structural features[139]
NetCBP	Network methods for predicting drug-target interactions. Furthermore, it suggests new drugs even when no data on their interactions with their targets are available[140]
DbMDR	Offers a database of multidrug resistance (MDR) genes and their orthologs, which could be used to develop new treatments [141]
TDR targets	Drug development molecular target identification and prioritization[142]
DrugBank	An extensive drug database with annotations covering drug targets and mechanisms of action[143]
PDTD	Database of potential proteins for *in silico* drug target identification[144]
DEG	Contains all known essential genes from different organisms[145]
TTD	Publicly accessible cross-links database that provides inclusive information about known therapeutic targets with related information, *i.e.* pathway information and the corresponding drugs/ligands[146]
KEGG	Offers information about the pathway, gene and ligands in three different databases, *i.e.* Pathway, Gene and Ligand[147]
Genecards	Officially known as Genecards: The Human Gene Database, it is an all-inclusive, authoritative compilation of annotative information about human genes[148]
DisGeNET	A public resource that houses a massive database of genetic variants and their links to human disease[149]
CTD	The Comparative Toxicogenomics Database is a vast, freely accessible database with the objective of increasing understanding of the effects of environmental exposures on human health. It includes information on chemical-gene/protein interactions, chemical-disease relationships and gene-disease links that has been curated by humans[150]
UniProt	The Universal Protein Resource is the world's most comprehensive, high-quality and freely accessible database of protein sequence and functional information[151]

SELF-BLM: Self-training bipartite local model; GEO: Gene Expression Omnibus; UniProt: Universal Protein Resource; CTD: Comparative Toxicogenomics Database; KEGG: Kyoto Encyclopedia of Genes and Genomes; TTD: Therapeutic Target Database; DEG: Database of Essential Genes; PDTD: Potential Drug Target Database; DbMDR: Database of multidrug resistance: NetCBP: Network-Consistency-based Prediction.

Keretsu *et al*[104] used computational methods to design new JAK1 inhibitors from a series of pyrrolopyridine derivatives. Autodock 4.2 was utilized to predict the protein-ligand binding. Tofacitinib, an established ligand, which was co-crystallized with the structure of JAK1 (protein data bank ID: 3EYG) obtained from the protein data bank (www.rcsb.org), was used as a reference compound to validate the docking procedure. The docked conformation of tofacitinib closely matched that of one obtained in the crystal structure. The binding affinity of compound 42 for JAK1 was found to be -10.2 kcal/mol. The F958 and L959 residues of the protein formed H-bond interactions with the pyrrolopyridine moiety. As a result, the methyl group of the methyl piperidine moiety protruded beyond the binding pocket and into the bulk solvent. The chlorobenzyl group slid into the hydrophobic pocket made by the residues of the activation loop, the α-helix and the P-loop. The selected binding conformation was prepared for further MD simulation study. The finding of the study was presented as a possible guide in the design of more effective JAK1 inhibitors[104].

In order to identify the bioactive compounds of the *Phyllanthus nivosus* leaf responsible for its activity against UC for further drug design, Johnson *et al*[101] combined molecular docking with an *in vivo* study. Levels of TNF-α, IL-6, nitric oxide, malondialdehyde, reduced glutathione, superoxide dismutase and catalase in the serum of rats experimentally induced with UC and treated with *Phyllanthus nivosus* were monitored. The bioactive ingredients of the most active fraction were identified using gas chromatography-mass spectrometry followed by molecular docking against IL-1b converting enzyme (caspase-1), beta-2 adrenergic receptor, cyclooxygenase-2 and TNF-α. The study led to the identification of ethyl iso-allocholate cholest-22-ene-21-ol, 3,5-dehydro-6-methoxy-, pivalate and alpha-cadinol as promising compounds for further development into drugs for the treatment of UC[101].

In another study, Halder *et al*[105] used molecular docking in combination with other *in silico* techniques for the repurposing of FDA-approved medications and provided a framework for drug exploration and computational methods in the discovery of drugs for the treatment of IBD. After being imported and processed using Protein Preparation Wizard and LigPrep, respectively, the molecular

Table 3 Molecular docking programs most frequently employed in drug discovery

Program	Description	Website
AutoDock [152]	A docking toolkit. It is meant to foretell the binding mode of small molecules to a receptor with a known 3D structure, such as a substrate or a drug candidate. There have been multiple engines developed, and it has undergone constant evolution and refinement over the years to incorporate new features	https://autodock.scripps.edu/
AutoDock Vina[153]	One of the AutoDock Suite's docking engines. It is a free and open-source molecular docking software. Dr. Oleg Trott of The Scripps Research Institute's Molecular Graphics Lab (now CCSB) created and initially implemented the system. The most recent version of AutoDock Vina is v.1.2.0	https://vina.scripps.edu/
Hex[154]	Invented by Dave Ritchie and is a program for molecular superposition and protein docking. Hex can read protein and DNA structures in the Protein Data Bank format as well as small-molecule SDF files. It has been downloaded over 40000 times as of December 2015	http://hex.loria.fr/
MOE[155]	Integrated computer-aided molecular design platform for small molecule and biological therapeutics. Common platform for chemists, biologists and crystallographers. Small Molecules - Peptides – Biologics	https://www.chemcomp.com/
Glide Schrodinger [156]	Provides a full range of speed *vs* accuracy options, ranging from the high-throughput virtual screening mode that efficiently enriches million compound libraries to the standard precision) mode that reliably docks tens to hundreds of thousands of ligands with high accuracy to the extra precision mode that eliminates false positives by sampling more extensively and using more advanced scoring, resulting in even higher enrichment	https://www.schrodinger.com/

CCSB: Center for Computational Structural Biology; SDF: Structure data files; DNA: Deoxyribonucleic Acid; MOE: Molecular operating environment.

target TNF (protein data bank ID: 2AZ5) with a small molecule inhibitor and the FDA-approved drugs (from the Zinc database) were subjected to molecular docking, ADMET analysis and binding free-energy calculation [Molecular mechanics with generalised Born and surface area solvation (MMGBSA)]. Following that, the medications were ranked based on docking score, ADMET parameters and MMGBSA dG binding score. The selected drugs were then subjected to an induced-fit docking study. The MD simulation study was conducted on the two most promising compounds, iopromide (ZINC000003830957) and deferoxamine (ZINC000003830635). Finally, the bioisosteric substitution was applied to enhance the ADMET properties of these compounds[105].

Pharmacophore modelling: Virtual screening of databases with the pharmacophore model has become one of the most important ways to find new lead compounds as compound databases and computing power have advanced. A pharmacophore is a conceptual description of the molecular features required for molecular recognition of a ligand by a biological macromolecule, which describes how structurally distinct ligands can bind to the same receptor site. To achieve therapeutic efficacy, drug molecules adopt an active conformation that is both geometrically and energetically complementary to that of the target macromolecule.

Medicinal chemists have discovered that modifications to specific chemical groups in drug molecules greatly affect the interaction between drugs and targets, while modifications to other groups have little to no effect[106]. Additionally, it was discovered that molecules exhibiting the same activity share common properties. Accordingly, Ehrlich proposed the idea of pharmacophores in 1909[107], which referred to the molecular framework of atoms with active essential characteristics. In 1977, Gund[108] provided further elaboration on the concept of pharmacophores as a class of molecules that recognize receptors and form structural features of molecular biological activity.

Pharmacophores can be discovered using one of two common approaches. If the structure of the target molecule is known, then the structure of the pharmacophore can be inferred using techniques like conformational analysis and molecular folding[109]. The pharmacophore recognition procedure will then choose an active compound that can be used to create the model. Conversely, pharmacophore studies are conducted on a number of compounds when either the structure of the target or its action mechanism is still unknown; this allows for a summary of data on certain groups that are crucial to the activity of the compound[24].

Babu *et al*[110] combined ligand-based pharmacophore modeling with virtual screening and molecular docking to find JAK1 inhibitors with high potency and selectivity. In the first step, they developed ligand-based pharmacophore models and checked them for accuracy with potency and selectivity validation techniques. A pharmacophore-based virtual screening was carried out on eight selected pharmacophore models using six different databases. ADME prediction and molecular docking were used to narrow down the hits found during screening. Docking results were verified using the binding free-energy calculation and induced fit docking techniques. A cross docking analysis was then performed to determine which lead compounds are selective for JAK1. In the end, five promising compounds were chosen and subjected to further investigation using MD and density functional theory. T5923555 and T5923531 were identified as the most promising leads among the five compounds and will be pursued for additional validation *via in vitro* and *in vivo* techniques.

QSAR: QSAR is frequently used in the drug discovery process to find compounds with desirable inhibitory effects on target proteins, with minimal side effects (nonspecific activity). The QSAR model is a quantitative investigation into the relationships that exist between small organic molecules and large biological macromolecules. The calculated properties of molecules (such as the absorption, distribution and metabolism of small organic molecules in living organisms) are correlated with their biological activity, as determined experimentally[111]. QSAR is the most precise and efficient approach to drug design when the structure of the receptor being targeted is unknown.

In the 1980s, 3D structural information was incorporated into the quantitative structure-activity relationship to form the 3D-QSAR method. Since the 1990s, structure-based drug design has increasingly replaced QSAR in the area of drug design due to the increase in computational power and the availability of the 3D structure of many biomolecules. However, QSAR with its advantages of small amount of calculation and good predictive ability[112], continues to play an important role in pharmaceutical studies.

3D-QSAR enables the investigation of the three-dimensional structure of bioactive compounds and the correct representation of energy changes and interaction patterns between bioactive molecules and receptors. Different drugs are evaluated by fitting their physicochemical and 3D structural parameters to the quantitative relationship. The structures of the newly created compounds are subsequently predicted and improved upon. 3D-QSAR analysis is a research method that integrates QSAR with computational chemistry and molecular graphics. It is an effective method for determining the nature of drug-target interactions, creating hypothetical images of simulated targets, determining the correlation between drug structure and activity and developing new medications. In addition, predictive 2D-QSAR models, 3D-QSAR models and 3D target and ligand-based approaches have all been developed for the purpose of finding IBD drugs[24].

Yang *et al*[113] incorporated molecular docking and QSAR study in the design of a series of TNF-α converting enzyme (TACE) inhibitors with the ability to bind in the S1′ pocket of the enzyme. A total of 12 analogues were synthesized by altering the chain length and alkylation pattern on the aromatic ring of the side chain. Most compounds inhibited cellular TNF-α production and TACE *in vitro*. The most promising compound from *in vitro* and *in vivo* pharmacokinetic studies had a moderate systemic clearance and a good oral bioavailability of 42%. It was also tested in a rat model of carrageenan-induced paw edema and found to be effective at reducing edema in the animal's paws. The series of α-alkoxyaryl alkyl substituted chromen-based analogues was then validated by means of a QSAR study and docking. Coumarin core TACE inhibitors with long, bulky α-substituent groups are able to enter the S1′ and S3′ pockets, where they form van der Waals interactions, with increased inhibitory activity. The docking study of the compound demonstrated its dual-function inhibitory activity toward TACE and matrix metallopeptidase-3. Based on the resulting QSAR descriptors, new α-substituted chromen-based TACE inhibitors with enhanced TACE inhibitory activity can be developed.

MD simulation in IBD drug discovery: MD simulation[102,114-116] is another popular approach to studying biomolecules; it is based on Newtonian mechanics and applies empirical molecular mechanics force fields. The drug discovery process can be aided by using explicit/implicit solvent models, which allow for simulations of time and space, and all-atom, united-atom and coarse-grained MD simulations [117,118]. MD simulations have typically been used to identify potential drug binding sites on target proteins, calculate the binding free energy between proteins and ligands, determine the mechanism of action of drug molecules, and more[119,120].

Taldaev *et al*[121] used MD to create structural representations of the arrangement of binding sites for the JAK family enzymes in order to elucidate the selectivity of upadacitinib for JAK1 among other isoforms. They found that the high affinity of upadacitinib was due to its ability to form four hydrogen bonds with amino acid residues in the hinge region of JAK1 as opposed to just two with other JAK isoforms. Structural features of the JAK1 binding site, including the unique residues S963 and E966, are responsible for stabilizing the molecule at the hinge region, as proposed by the authors. Hydrogen bonding with the JAK1 (E883) and JAK2 (N859) amino acid residues in the glycine loops was reported to increase the affinity. The research findings were presented as having the potential to direct the creation of more selective and effective next-generation JAK inhibitors, thereby enhancing the treatment of a wide range of cytokine-mediated diseases.

In another MD simulation research conducted by Du *et al*[122], the interaction mechanism between oncostatin M (OSM) and its receptor (OSMR) at the atomic level was predicted. Binding of OSM to OSMR is said to be implicated in the pathogenesis of IBD. The OSMR interaction domain was built using the homology modeling approach. Docking was used to establish the near-native structure of the OSM-OSMR complex, and long-time scale MD simulation in an explicit solvent was used to sample the conformations when OSM binds to OSMR. Following the equilibration of the simulated system, the per-residue energy contribution was determined to describe the key residues for the formation of the OSM-OSMR complex. Premised on these key residues, eight residues (OSM: Arg100, Leu103, Phe160, and Gln161; OSMR: Tyr214, Ser223, Asp262 and Trp267) were identified as "hot spots" by computational alanine mutagenesis analysis and confirmed by further MD simulation of the R100A (one of the discovered "hotspots") mutant. Furthermore, the FTMap analysis revealed six cavities at the OSM-

OSMR interface, which were proposed as key binding sites. The predicted 3D structure of the OSM-OSMR complex and the discovered "hotspots" provide useful information in understanding the OSM-OSMR interactions, and the identified locations serve as potential targets in designing small molecules to inhibit the interactions[122].

CONCLUSION

The global prevalence of IBD is increasing, with developing countries experiencing an increase due to modernization. There is still a need for a new generation of alternative therapies due to the loss of response to biologic drugs, which can be caused in part by the immunogenicity of the administered protein as well as the need to discontinue drugs due to intolerance or side effects. The development of small molecule drugs is difficult because of the complexity of the molecular pathways involved in the progression of disease. The conventional drug design and development processes are lengthy, expensive and filled with arduous scientific procedures. However, computational tools show considerable promise as a practical means of creating new small molecules with biomedical application. There have been a number of groundbreaking successes with drugs for several diseases developed using CADD. Many of these drugs are either FDA-approved or under clinical trials. However, computational methods appear to be underutilized for IBD drug research, as observed during this literature search. To hasten efforts in finding treatments for IBD, more scientists need to turn to computational methods. The modern drug discovery process for IBD makes use of a wide range of computational tools, some of which are geared toward specific tasks such as genomic studies, target identification, and virtual screening.

FOOTNOTES

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Country/Territory of origin: Nigeria

ORCID number: Titilayo Omolara Johnson 0000-0002-4230-8025.

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Siriporn Chamniansawat, Nasisorn Suksridechacin, Narongrit Thongon

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Siriporn Chamniansawat, Division of Anatomy, Department of Medical Sciences, Faculty of Allied Health Sciences, Burapha University, Muang 20131, Chonburi, Thailand

Nasisorn Suksridechacin, Biodiversity Research Centre, Thailand Institute of Scientific and Technological Research, Khlong Luang 12120, Pathum Thani, Thailand

Narongrit Thongon, Division of Physiology, Department of Medical Sciences, Faculty of Allied Health Sciences, Burapha University, Muang 20131, Chonburi, Thailand

Corresponding author: Narongrit Thongon, PhD, Associate Professor, Division of Physiology, Department of Medical Sciences, Faculty of Allied Health Sciences, Burapha University, 169 Long-Hard Bangsaen Road, Saensook, Muang 20131, Chonburi, Thailand.
narongritt@buu.ac.th

Abstract

Magnesium (Mg^{2+}) has an important role in numerous biological functions, and Mg^{2+} deficiency is associated with several diseases. Therefore, adequate intestinal absorption of Mg^{2+} is vital for health. The small intestine was previously thought to absorb digested Mg^{2+} exclusively through an unregulated paracellular mechanism, which is responsible for approximately 90% of total Mg^{2+} absorption. Recent studies, however, have revealed that the duodenum, jejunum, and ileum absorb Mg^{2+} through both transcellular and paracellular routes. Several regulatory factors of small intestinal Mg^{2+} uptake also have been explored, *e.g.*, parathyroid hormone, fibroblast growth factor-23, apical acidity, proton pump inhibitor, and pH-sensing channel and receptors. The mechanistic factors underlying proton pump inhibitor suppression of small intestinal Mg^{2+}, such as magnesiotropic protein dysfunction, higher mucosal bicarbonate secretion, Paneth cell dysfunction, and intestinal inflammation, are currently being explored. The potential role of small intestinal microbiomes in Mg^{2+} absorption has also been proposed. In this article, we reviewed the current knowledge on the mechanisms and regulatory factors of small intestinal Mg^{2+} absorption.

Key Words: Hormone; Magnesium absorption; Paneth cells; Proton pump inhibitor; Regulation; Small intestine

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Core Tip: Small intestinal epithelium absorbs digested magnesium (Mg^{2+}) through both transcellular active and paracellular passive mechanisms. Several regulatory factors of small intestinal Mg^{2+} uptake have been reported. Parathyroid hormone and fibroblast growth factor-23 directly inhibit transcellular Mg^{2+} absorption in the duodenum, jejunum, and ileum. The apical proton triggers acid-sensing ion-channel 1a and purinergic $P2Y_2$ receptor activities, which stimulates mucosal bicarbonate secretion and induces $MgCO_3$ precipitation to suppress absorption. Omeprazole suppresses Mg^{2+} absorption in the duodenum, jejunum, and ileum.

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INTRODUCTION

Magnesium (Mg^{2+}) has an essential role in numerous cellular biochemical functions ranging from DNA structure stability and repairing, cell proliferation, neuronal excitability, bronchodilatation, vasodilatation, muscle contraction, myocardial excitability, bone hydroxyapatite formation, and anti-inflammatory function to exocrine and endocrine function of the pancreas[1]. Mg^{2+} deficiency has been implicated in several diseases, such as Alzheimer's disease[2], osteoporosis[3], hypertension[4], diabetes mellitus[5], and cancer[6]. Therefore, its plasma level is tightly regulated within a narrow range (0.7–1.1 mmol/L) by the collaborative actions of intestinally digested Mg^{2+} absorption, bone and muscle Mg^{2+} storage, and excess renal Mg^{2+} excretion[1]. The mechanism underlying regulation of transepithelial Mg^{2+} transport has been extensively explored in the renal tubular epithelium[1]. However, few research articles on the mechanism and regulatory factors of intestinal Mg^{2+} absorption have been published.

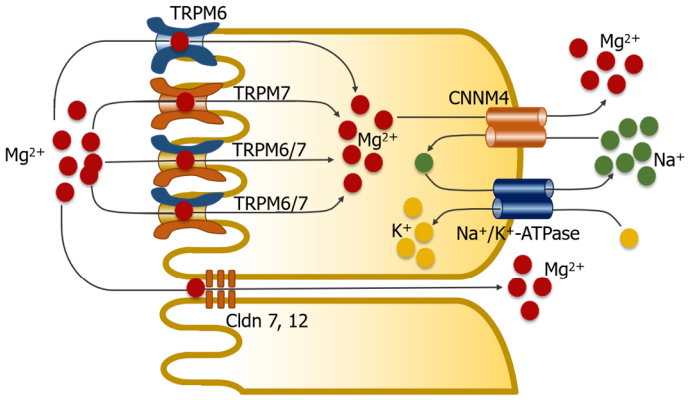
Since dietary intake is the sole source of Mg^{2+} in humans, adequate intestinal absorption of Mg^{2+} is vital for normal Mg^{2+} balance. It was previously hypothesized that bulk Mg^{2+} uptake occurs in the small intestine through an unregulated paracellular pathway, whereas fine-tuning of colonic Mg^{2+} absorption occurs through a regulated transcellular mechanism[1,7,8]. Colonic Mg^{2+} absorption can be modulated by dietary Mg^{2+} content and inulin fibers[7,9] but not by hormones[1,7]. In contrast, recent studies have provided new insights into the mechanisms and modulatory factors of small intestinal Mg^{2+} uptake. The aim of this article was to review the current knowledge of the mechanisms and regulatory factors of small intestinal Mg^{2+} absorption.

MECHANISM OF SMALL INTESTINAL MG^{2+} ABSORPTION

The mechanism of small intestinal Mg^{2+} absorption is currently under debate. One research group has proposed that transient receptor potential melastatin 6 homodimer channel (*TRPM6*) mRNA expression and transcellular Mg^{2+} absorption were not present in the small intestine[1,7,8]. However, a study from the same group showed positive immunofluorescence staining of TRPM6 protein in the absorptive cells along the brush border membrane of the villi in the duodenum[10]. Another group has proposed that the small intestinal epithelium absorbs Mg^{2+} through transcellular active and paracellular passive transport mechanisms[11-13]. In an Ussing chamber study, transport of transcellular and paracellular Mg^{2+} was detected in the duodenum, jejunum, and ileum[11-13]. The proposed mechanism of small intestinal Mg^{2+} absorption is shown in Figure 1.

Transcellular Mg^{2+} absorption

Transcellular Mg^{2+} absorption occurs through mucosal Mg^{2+} uptake by TRPM6 and TRPM7 homodimer channel, both of which were markedly detected in the small intestinal epithelium of human and murine cells[10-14]. In addition, recent mass spectrometric peptide sequence analysis confirmed the expression of TRPM6 and TRPM7 in the duodenum and jejunum[15]. The channel activities of both homodimers of TRPM6 and of TRPM7 are negatively regulated by physiological Mg·ATP and Mg^{2+} levels[10,16-19]. A recent study reported the expression of a heterodimer TRPM6/7 channel in the plasma membrane of duodenal and jejunal epithelium[15]; therefore, Mg^{2+} enters the small intestinal epithelial cells through TRPM6/7, TRMP6, and TRPM7. However, the heterodimer TRPM6/7 channels do not respond to physiological intracellular Mg^{2+} and Mg·ATP[17,19]; thus, continuous epithelial Mg^{2+} absorption can occur through the TRPM6/7 channel, regardless of intracellular Mg^{2+} and concentrations. Basolateral Mg^{2+} extrusion from the small intestinal epithelium occurs through cystathionine β-synthase domain divalent metal cation transport mediator 4[11-13,20] by means of a sodium (Na^+) gradient-dependent



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Figure 1 Magnesium absorption in the small intestine through two absorption pathways. The transcellular transport mechanism involves magnesium (Mg^{2+}) influx into enterocytes through the transient receptor potential melastatin 6 homodimer channel (TRPM6), transient receptor potential melastatin 7 homodimer channel (TRPM7), and transient receptor potential melastatin 6/7 heterodimer channel (TRPM6/7). Cystathionine β-synthase domain divalent metal cation transport mediator 4 (CNNM4) mediates basolateral Mg^{2+} extrusion by means of secondary active transport. In the paracellular mechanism, Mg^{2+} moves through tight-associated paracellular pores of Claudin 7 (Cldn 7) and Claudin 12 (Cldn 12).

secondary active transport[20]. However, mutation of cystathionine β-synthase domain divalent metal cation transport mediator 4 does not affect the plasma concentration in humans[21,22], suggesting that other Mg^{2+} extrusion mechanisms probably occur.

Paracellular Mg^{2+} absorption

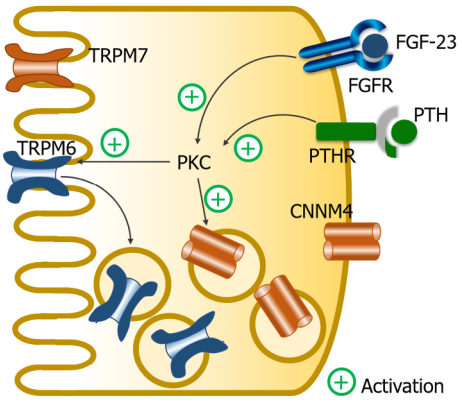
It has been suggested that paracellular Mg^{2+} absorption is responsible for 90% of total intestinal Mg^{2+} uptake[23]. Paracellular permeability is regulated by the paracellular claudin (Cldn) channel of the tight junction[24]. In 1999, the first discovery of a paracellular channel at the tight junction was Cldn-19 or paracellin-1, which form a paracellular Mg^{2+} channel[25]. It is thought that paracellular Mg^{2+} channels in epithelial tissues are formed by Cldn-16 and -19[25-27]; mutations in these genes lead to severe hypomagnesemia. The small intestinal epithelium expresses Cldn-1–5, -7, -8, -12, and -15 but not -16 and -19[28,29]. A previous study proposed that Cldn-7 and -12 modulated intestinal paracellular Mg^{2+} absorption[30]. However, the processes involving Cldn-regulated paracellular Mg^{2+} absorption in the small intestine still must be elucidated.

REGULATORY FACTORS OF SMALL INTESTINAL MG^{2+} ABSORPTION

Hormones

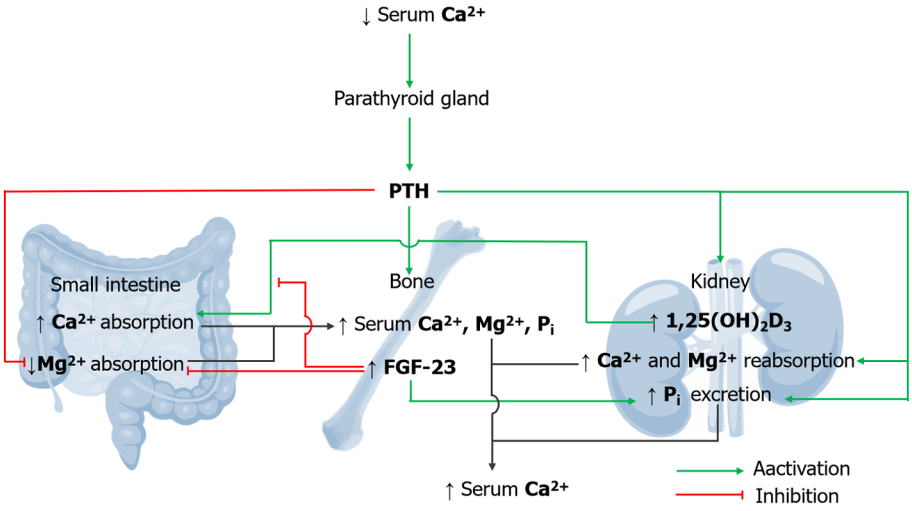
In general, hormones mainly modulate the transcellular electrolyte transport to regulate epithelial electrolyte absorption or secretion. Hormonal regulation of small intestinal Mg^{2+} absorption also modulates transcellular Mg^{2+} absorption. A recent study reported that parathyroid hormone (PTH) and fibroblast growth factor-23 (FGF-23) systemically and directly inhibited transcellular, but not paracellular, Mg^{2+} absorption in the duodenum, jejunum, and ileum[13]. There was no additional effect of PTH and FGF-23, suggesting that they acted through the same intracellular signaling molecule. Both PTH and FGF-23 activate their corresponding receptors that further stimulate the same protein kinase C pathway to suppress plasma membrane-associated TRPM6 expression (Figure 2). Since native TRPM6 primarily functions as a subunit of heteromeric TRPM6/7 channels[31], the suppression of plasma membrane TRPM6 probably suppresses plasma TRPM6/7 heterodimer expression. The suppression of plasma TRPM6 and TRPM6/7 activity leads to diminution of transcellular Mg^{2+} absorption[13]. The inhibitory effect of PTH and FGF-23 could be nullified by Gö 6850[13], which inhibits the conventional (α, β1, β2, and γ) and novel (δ and ε) protein kinase C isoforms. However, the exact signaling pathway of PTH and FGF-23 inhibition of small intestinal transcellular Mg^{2+} absorption requires further study.

The proposed physiologically relevant magnesiotropic actions of PTH and FGF-23 are shown in Figure 3. During hypocalcemia, the parathyroid gland actively secretes PTH into the blood stream. PTH stimulates the bone resorption process, which increases plasma calcium (Ca^{2+}), inorganic phosphate (Pi), and Mg^{2+} levels[32,33]. PTH stimulates renal 1,25-dihydroxy vitamin D_3 [$1,25(OH)_2D_3$] production, which subsequently induces small intestinal Ca^{2+} absorption[34]. PTH also activates renal tubular Ca^{2+} and Mg^{2+} reabsorption[32]. Plasma Pi and PTH trigger bone-derived FGF-23 release, which acts as a negative feedback regulator to abolish $1,25(OH)_2D_3$-induced intestinal Ca^{2+} absorption[33]. PTH and FGF-23 synergistically suppress the small intestinal absorption of dietary Mg^{2+}[13] to prevent hypermagnesemia. PTH and FGF-23 downregulate the Na^{2+}-dependent Pi cotransporters, (NaPi)-IIa and NaPi-IIc,



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Figure 2 Fibroblast growth factor-23 and parathyroid hormone regulate magnesium absorption in the small intestine. Fibroblast growth factor-23 (FGF-23) and parathyroid hormone (PTH) act through their corresponding receptors to suppress magnesium absorption in the protein kinase C (PKC)-dependent pathway; they suppressed membrane transient receptor potential melastatin 6 homodimer channel (TRPM6) expression, which leads to the suppression of membrane transient receptor potential melastatin 6/7 (TRPM6/7) expression. FGF-23 and PTH also increase cytosolic cystathionine β-synthase domain divalent metal cation transport mediator 4 (CNNM4) expression. TRPM7: Transient receptor potential melastatin 7 homodimer channel; FGFR: Fibroblast growth factor receptor; PTHR: Parathyroid hormone receptor.



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Figure 3 Integrated magnesiotropic action of fibroblast growth factor-23 and parathyroid hormone. Parathyroid hormone (PTH) stimulates the bone resorption process, which increases plasma calcium (Ca^{2+}), inorganic phosphate (P_i), and magnesium (Mg^{2+}) levels. PTH stimulates the release of 1,25-dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) to subsequently induce small-intestinal Ca^{2+} absorption. PTH also activates renal tubular Ca^{2+} and Mg^{2+} reabsorption. Plasma Pi and PTH trigger the release of fibroblast growth factor-23 (FGF-23) to abolish $1,25(OH)_2D_3$-induced intestinal Ca^{2+} absorption. PTH and FGF-23 synergistically suppress small intestinal absorption of dietary Mg^{2+}. PTH and FGF-23 induce urinary P_i excretion.

and increase urinary Pi excretion[32] to prevent hyperphosphatemia. Therefore, PTH and FGF-23 exert their calcemic effect by preventing hyperphosphatemia and hypermagnesemia.

Luminal acidity

The hypothesis that apical acidity and mucosal bicarbonate secretion (MBS) affect luminal Mg^{2+} solubility and intestinal Mg^{2+} absorption was previously proposed in 2014[11,35], which was confirmed in a recent review article[36]. The luminal acidity along the entire human and rodent small bowel varies from pH 5.0–7.3[12,37]. The luminal protons provide an appropriate environment for mineral absorption by stabilizing their ionized forms[38]. The elevation of luminal pH led to a lower soluble Mg^{2+}, which decreased from 79.61% of total luminal Mg content at pH 5.15% to 8.71% of total luminal Mg at pH 7.8[39]. Therefore, luminal acidity enhances Mg^{2+} absorption in the human small intestine[40] and epithelial-like Caco-2 monolayers[30,35]. The MBS and luminal pH elevation diminished duodenal, jejunal, and ileal Mg^{2+} absorption[11,12].

pH-sensing channel and receptor

Small intestinal enterocytes are regularly exposed to strong gastric acid. When luminal protons are present in the duodenal lumen, the intestinal epithelium cells can directly detect and modulate their cellular response through the proton-sensing channels, *e.g.*, the acid-sensing ion-channel 1a (ASIC1a) or proton-sensing receptors, such as ovarian cancer G protein-coupled receptor 1 (OGR1) and $P2Y_2$ purinoceptor[41-44].

OGR1, also known as GPR68, is expressed in the human small intestine, spleen, testes, brain, lungs, placenta, heart, and kidneys but not in the colon[44]. OGR1 is a proton-sensitive receptor with pH values at half activation ($pH_{0.5}$) and full activation of 7.2 and 6.8, respectively[45-47]. When the luminal pH decreases to 6.5, OGR1 activity is inactivated[45]. Activation of OGR1 triggers the phospholipase C–protein kinase C signaling pathway to activate intestinal Mg^{2+} absorption[35] (Figure 4).

ASIC1a is a proton-sensitive Ca^{2+} channel with a $pH_{0.5}$ of 6.2[41,43]. Activation of ASIC1a activates intracellular Ca^{2+} signaling and subsequently induces MBS. In the intestinal epithelium, luminal proton stimulates ASIC1a activity that further activates MBS in a Ca^{2+} signaling-cystic fibrosis transmembrane conductance regulator-dependent mechanism[35] (Figure 4). Secreted bicarbonate has previously been found to reduce luminal protons[48] and induce precipitation of luminal free Mg^{2+}[49], thus reducing free soluble Mg^{2+} and suppressing intestinal Mg^{2+} absorption.

Purinergic regulation of luminal pH and electrolyte transport in the small intestine have been described[50-52]. Duodenocytes regularly secrete ATP into its lumen. If luminal pH is low, luminal alkaline phosphatase activity is diminished and luminal ATP increases, which subsequently activates $P2Y_2$ purinoceptor. Simultaneously, $P2Y_2$ is a proton-sensitive receptor that is activated by luminal protons[42]. Active $P2Y_2$ purinoceptors further activate MBS to increase luminal pH. A previous study showed that $P2Y_2$ activation induced MBS through a cystic fibrosis transmembrane conductance regulator- and Na^+-HCO_3^- cotransporter-1-dependent mechanism, which subsequently suppressed intestinal Mg^{2+} absorption[53] (Figure 5).

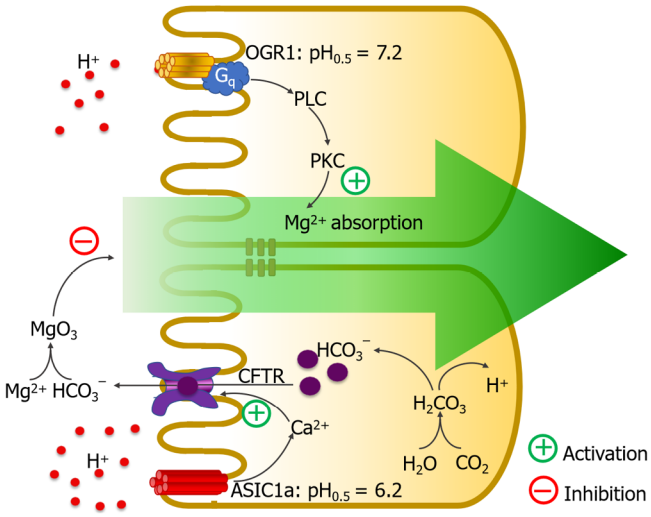
Proton pump inhibitor

Proton pump inhibitor (PPI)-induced hypomagnesemia (PPIH) and hypomagnesuria in humans have been reported since 2006[54-57]. Intravenous Mg^{2+} supplementation or withdrawal of the PPI was able to rapidly normalize plasma and urinary Mg^{2+} levels in PPIH patients, though oral Mg^{2+} supplementation could not. Clinical assessments have reported that PPIH patients had normal renal Mg^{2+} handling[54,56,57]. These findings suggest that PPIs could suppress intestinal Mg^{2+} absorption. Our group has extensively studied the underlying mechanisms of PPI-suppressed intestinal Mg^{2+} absorption for a decade[11,12,15,30,35,53,58,59]. Our results suggest that PPIs mainly suppressed small intestinal Mg^{2+} absorption.

Omeprazole, the first introduced PPI, significantly suppressed total, transcellular, and paracellular Mg^{2+} absorption in the duodenum, jejunum, ilium, and colon of PPIH rats[11,12]. Regarding the percent suppression of total Mg^{2+} absorption in the duodenum (81.86%), jejunum (70.59%), ileum (69.45%), and colon (39.25%), the small intestine is the segment most adversely affected by prolonged PPI administration. However, previous articles have proposed that PPIs mainly inhibit colonic Mg^{2+} absorption[36,60,61], but those study results remain controversial[60,61]. They also proposed that colonic fermentation of dietary fibers probably increased serum Mg^{2+} and cured patients with PPIH[36]. A previous study clearly showed that dietary inulin fibers significantly induced cecal and colonic fermentation, but not plasma Mg^{2+} levels, in control and PPIH mice[61]. In contrast, dietary inulin fibers significantly induced renal Mg^{2+} excretion in PPIH mice[61], which should aggravate hypomagnesemia in PPIH. Therefore, the large intestine may not be a suitable intestinal segment that should be modulated to counteract PPIH.

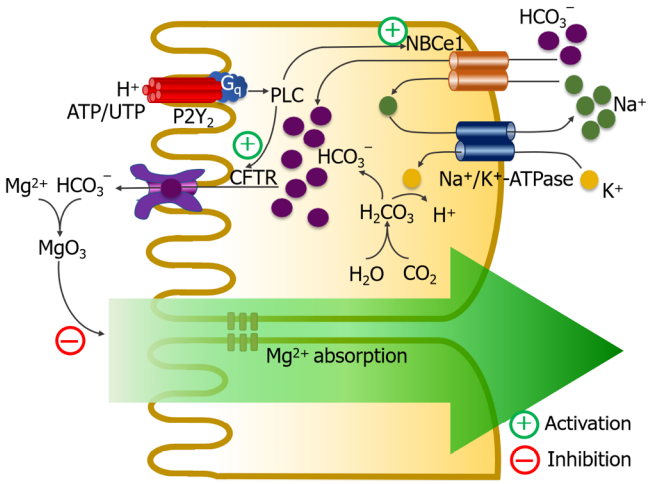
The proposed mechanism of PPI-suppression of small intestinal Mg^{2+} absorption is shown in Figure 6. PPIs markedly suppress membranous TRPM7 and TRPM6/7[15]. Membranous TRPM6-channel activity is suppressed by hyperphosphorylation at the T1851 residue and hyperoxidation at the M1755 residue [15]. Phosphorylation of the T1851 residue of the TRPM6 protein induces TRPM6-channel suppression by intracellular free Mg^{2+} and activated 5 C-kinase 1[62]. Oxidation of the M1755 residue in the TRPM6 protein also suppresses its channel permeability[63]. Suppression of membranous TRPM6, TRPM7, and TRPM6/7 disrupts mucosal Mg^{2+} entry into the small intestinal epithelium and then inhibits transcellular Mg^{2+} absorption[11,12]. Plasma FGF-23 was markedly increased in PPIH rats[12]. The mechanism by which FGF-23 inhibits transcellular small intestinal Mg^{2+} absorption is described in the above section[13]. Therefore, PPI-suppressed transcellular Mg^{2+} absorption is due, at least in part, to FGF-23.

PPIs suppress paracellular Mg^{2+} absorption (Figure 6). The small intestinal epithelium only expresses Cldn-1, -2, -3, -4, -5, -7, -8, -12, and -15[28,29]. Overexpression of Cldn proteins and higher paracellular resistance have been demonstrated in the small intestines of PPIH rats[11,12]. Paracellular tight junction width was significantly decreased in the small intestine of PPIH rats[58]. PPIs also suppress epithelial paracellular Mg^{2+} permeability and cation selectivity[30,59]. These results shed light on the mechanism of PPI-suppressed paracellular Mg^{2+} absorption in the small intestine.



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Figure 4 Ovarian cancer G protein-coupled receptor 1 and acid-sensing ion-channel 1a modulate intestinal magnesium absorption. Activation of ovarian cancer G protein-coupled receptor 1 (OGR1) triggers the phospholipase C (PLC)–protein kinase C (PKC) signaling pathway to activate intestinal magnesium (Mg^{2+}) absorption. Activation of acid-sensing ion-channel 1a (ASIC1a) activates intracellular calcium (Ca^{2+}) signaling to induce mucosal bicarbonate secretion in a cystic fibrosis transmembrane conductance regulator (CFTR)-dependent mechanism. Secreted bicarbonate (HCO_3^-) reduces free soluble Mg^{2+} and suppresses intestinal Mg^{2+} absorption. H: Hydrogen.

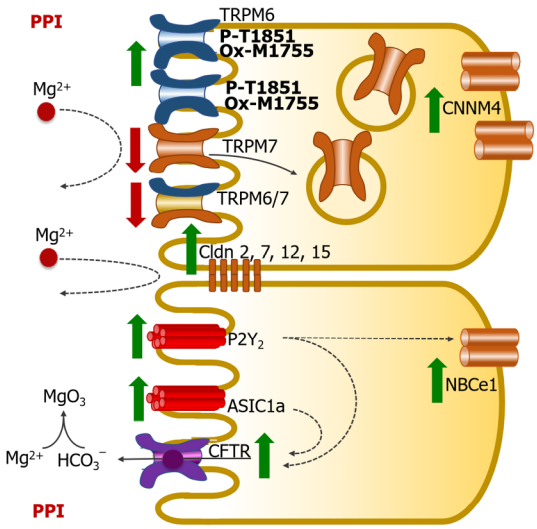


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Figure 5 $P2Y_2$ purinoceptors modulate intestinal magnesium absorption. Activation of $P2Y_2$ purinoceptor stimulates luminal cystic fibrosis transmembrane conductance regulator and basolateral Na^+-HCO_3^- cotransporter-1 (NBCe1) activities through a phospholipase C (PLC)-dependent mechanism. Active cystic fibrosis transmembrane conductance regulator (CFTR) and Na^+-HCO_3^- cotransporter-1 induce mucosal bicarbonate (HCO_3^-) secretion, which reduces luminal free magnesium (Mg^{2+}) and suppresses Mg^{2+} absorption. Ca^{2+}: Calcium; H: Hydrogen; K: Potassium; Na^+: Sodium.

PPI-induced small intestinal MBS (Figure 6) has been reported in humans[64], PPIH rats[11], and PPI-treated Caco-2 monolayers[35,53]. PPIs have also been shown to significantly increase ASIC1a and $P2Y_2$ expression in PPI-treated epithelium[35,53]. Active ASIC1a and $P2Y_2$ trigger MBS. Higher secreted bicarbonate in PPIH small intestines reduces free soluble Mg^{2+}, which disrupts Mg^{2+} absorption (Figure 6). Inhibition of MBS significantly increases duodenal Mg^{2+} absorption in PPIH rats[11].

In addition to the change in magnesiotropic protein expression and function and MBS, PPIs have been shown to induce structural change in the absorptive epithelium of the small intestine[58]. Prolonged PPI administration markedly decreased the villous length and absorptive area in the duodenal, jejunal, and ilial epithelium of PPIH rats. The underlying mechanism involves Paneth cell dysfunction in the small intestine[58]. Paneth cells have an important role in host-microorganism homeostasis in the small intestine by providing antimicrobial α-defensin peptides[65,66]. Disruption of the secretory function of Paneth cells leads to infection and chronic inflammation of the small intestine [65,66]. In PPIH rats, a reduction in secretory granules and metaplasia of Paneth cells occurs in the duodenum, jejunum, and ileum, suggesting Paneth cell secretory dysfunction[58]. Chronic inflam-



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Figure 6 Mechanism of proton pump inhibitor-suppressed small intestinal magnesium absorption in proton pump inhibitor-induced hypomagnesemia rats. Proton pump inhibitor (PPI) suppresses membrane transient receptor potential melastatin 7 homodimer channel (TRPM7) and transient receptor potential melastatin 6/7 homodimer channel (TRPM6/7) expression but increases membrane transient receptor potential melastatin 6 homodimer channel (TRPM6) and cystathionine β-synthase domain divalent metal cation transport mediator 4 (CNNM4) expression. PPI induces phosphorylation of the T1851 residue and oxidation of the M1755 residue of the membrane TRPM6 channel, which reduces their channel permeability. These PPI effects reduce transcellular magnesium (Mg^{2+}) absorption. Overexpression of claudin 2 (Cldn2), claudin 7 (Cldn7), claudin 12 (Cldn12), and claudin 15 (Cldn15) reduces paracellular permeability, which suppresses paracellular Mg^{2+} absorption. PPI also enhances $P2Y_2$- and acid-sensing ion-channel 1a (ASIC1a)-suppressed intestinal Mg^{2+} absorption. CFTR: Cystic fibrosis transmembrane conductance regulator; HCO_3^-: Bicarbonate; NBCe1: Na^+-HCO_3^- cotransporter-1.

mation in the small intestinal epithelium leads to villous atrophy and reduction of the absorptive area in the small intestine of PPIH rats[58].

Gut microbiota

The potential role of gut microbiota in colonic Mg^{2+} absorption has previously been proposed[36]. However, it is currently unknown how the small intestinal microbiome affects small intestinal Mg^{2+} absorption. Previous studies have shown that the small intestine is colonized by a complex gut microbiota community and is less numerous and diverse (approximately 10^3–10^7 microbial cells/gram) than in the colon (approximately 10^{12} microbial cells/gram)[67]. The dominant bacterial phyla in the small intestine are *Streptococcus sp*., *Lactobacillaceae*, and *Enterobacteriaceae*, whereas in the colon, the dominant phyla are *Bacteroidaceae, Prevotellaceae, Rikenellaceae, Lachnospiraceae*, and *Ruminococcaceae*[68,69]. Prolonged PPI treatment can lead to gut microbiota dysbiosis, such as the reduction of *Actinobacteria* and *Bifidobacteria spp*., which are responsible for maintaining the mucosal barrier function[68].

Furthermore, long-term treatment with PPIs causes small intestinal bacterial overgrowth because of the loss of the gastric acid defensive barrier[70]. The jejunal samples of small intestinal bacterial overgrowth patients regularly showed increased production of toxic agents, such as serum endotoxin and bacterial compounds that stimulate the secretion of proinflammatory cytokines[71]. Apart from these findings, our previous study showed Paneth dysfunction and chronic inflammation in the small intestine of PPIH rats[58]. From the perspective of relevant gut microbiota, Paneth cell defects have been found to be associated with increased *Bacteroidetes* and *Enterococcus* and decreased *Bifidobacterium*[72], whereas *Bifidobacterium longum* has been found to promote cell proliferation and expression of Lgr5 and Wnt3a in intestinal organoids and alleviate microbiota dysbiosis by regulating the functions of Paneth cells[73]. It is also possible that the synthesis of gut microbiota metabolites could lead to changes in the absorptive surface in the gut and/or stimulate gene expression[74].

In the colon, bifidobacterial fermentation leads to acidification of the colon, which shows beneficial absorption of Mg^{2+}[9,61,75]. In humans, small intestinal microbiota can also ferment the available carbohydrates and induce intestinal acidification[76]. In the human small intestine, a dominant bacterial phylum is *Streptococcus sp*.[77,78], which is an anaerobe that can ferment relatively simple carbohydrates at a high rate[79]. According to the above, luminal acidity markedly induces small intestinal Mg^{2+} absorption. Therefore, small intestinal fermentation should induce small intestinal Mg^{2+} absorption.

CONCLUSION

Bulk absorption of digested Mg^{2+} occurs in the small intestine through transcellular active and

paracellular passive mechanisms. PTH, FGF-23, luminal protons, ASIC1a, OGR1, $P2Y_2$, PPIs, and the microbiome have recently been proposed as regulatory factors of small intestinal Mg^{2+} uptake. However, the regulatory mechanism of small intestinal Mg^{2+} requires additional extensive studies.

FOOTNOTES

Author contributions: Chamniansawat S wrote the manuscript; Suksridechacin N wrote the manuscript; Thongon N wrote and edited the manuscript.

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Country/Territory of origin: Thailand

ORCID number: Siriporn Chamniansawat 0000-0003-2609-6373; Nasisorn Suksridechacin 0000-0002-0236-6650; Narongrit Thongon 0000-0003-2475-9161.

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MINIREVIEWS

Hepatocellular carcinoma in non-alcoholic steatohepatitis without cirrhosis

Cristiane Valle Tovo, Angelo Zambam de Mattos, Gabriela Perdomo Coral, Giovana D P Sartori, Livia Villela Nogueira, Gustavo Tovo Both, Cristiane A Villela-Nogueira, Angelo A de Mattos

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Cristiane Valle Tovo, Angelo Zambam de Mattos, Gabriela Perdomo Coral, Giovana D P Sartori, Angelo A de Mattos, Department of Internal Medicine, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre 90050170, RS, Brazil

Livia Villela Nogueira, Department of Internal Medicine, Fundação Técnico Educacional Souza Marques, RJ 21491-630, RJ, Brazil

Gustavo Tovo Both, Department of Internal Medicine, Universidade Luterana do Brasil, Canoas 92425-350, RS, Brazil

Cristiane A Villela-Nogueira, Department of Internal Medicine, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21491-630, RJ, Brazil

Corresponding author: Cristiane Valle Tovo, MD, PhD, Associate Professor, Internal Medicine, Universidade Federal de Ciências da Saúde de Porto Alegre, Rua Sarmento Leite 245, Porto Alegre 90050170, RS, Brazil. cris.tovo@terra.com.br

Abstract

Cirrhosis is an emerging major cause of the development of hepatocellular carcinoma (HCC), but in non-alcoholic fatty liver disease (NAFLD), up to 50% of patients with HCC had no clinical or histological evidence of cirrhosis. It is currently challenging to propose general recommendations for screening patients with NAFLD without cirrhosis, and each patient should be evaluated on a case-by-case basis based on the profile of specific risk factors identified. For HCC screening in NAFLD, a valid precision-based screening is needed. Currently, when evaluating this population of patients, the use of non-invasive methods can guide the selection of those who should undergo a screening and surveillance program. Hence, the objective of the present study is to review the epidemiology, the pathophysiology, the histopathological aspects, the current recommendations, and novel perspectives in the surveillance of non-cirrhotic NAFLD-related HCC.

Key Words: Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Hepatocellular carcinoma; Genetic variants; Microbiota; Obesity

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Core Tip: Cirrhosis is an emerging major cause of the development of hepatocellular carcinoma (HCC), but in non-alcoholic fatty liver disease (NAFLD), up to 50% of patients with HCC had no clinical or histological evidence of cirrhosis. In the present study, we evaluated data regarding the epidemiology, the pathophysiology, the histopathological aspects, the current recommendations, and novel perspectives in the surveillance of non-cirrhotic NAFLD-related HCC. We believe that using non-invasive methods can guide the selection of patients who need to undergo screening and a surveillance program.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has been traditionally defined by the evidence of hepatic steatosis by imaging or histology and by the lack of secondary causes of hepatic fat accumulation such as significant alcohol consumption, long-term use of steatogenic medications, hereditary disorders and other causes of chronic liver diseases[1]. Recently, there has been a proposal to rename NAFLD to metabolic associated fatty liver disease (MAFLD), thus eliminating the need to exclude other causes of liver diseases and adopting inclusive criteria according to coexistence with other liver diseases[2]. The diagnosis of MAFLD is based on histological, imaging or blood biomarker evidence of fat accumulation in the liver (steatosis) in addition to one of the following criteria, namely overweight/obesity, type 2 diabetes mellitus (DM) or evidence of metabolic dysregulation[2].

NAFLD is a well-known cause of chronic liver disease, compromising more than 25% of the global population, and up to 25% may have nonalcoholic steatohepatitis (NASH) with or without fibrosis. NASH with fibrosis is the most active form of disease which is associated with significant morbidity and mortality due to complications of liver cirrhosis, hepatic decompensation, and hepatocellular carcinoma (HCC)[3].

Cirrhosis is an emerging major cause of the development of HCC, but in NAFLD, up to 50% of patients with HCC had no clinical or histological evidence of cirrhosis[4-6].

NAFLD and the components of metabolic syndrome, especially obesity and DM, are independently associated with HCC development and contribute to the risk of HCC in a non-cirrhotic liver[7,8]. Non-cirrhotic NAFLD patients have a 2.5-fold higher risk of developing HCC than other etiologies of chronic liver disease without cirrhosis[9].

The objective of the present study is to review the epidemiology, the pathophysiology, the histopathological aspects, the current recommendations and novel perspectives in the surveillance of non-cirrhotic NAFLD-related HCC.

EPIDEMIOLOGY OF NAFLD-RELATED HCC

Liver cancer, most of which corresponds to HCC[10,11], ranks sixth among the most common malignancies and second among the leading causes of cancer-related death worldwide. HCC affected 11.6/100000 individuals in 2020, leading to a mortality rate of 10.7/100000[12]. Remarkably, despite major advances in the treatment of viral hepatitis, it is estimated that the incidence rate of liver cancer will keep increasing until 2030, which can be partly explained by a striking increase in the incidence of NAFLD-related HCC[13].

NAFLD affects approximately one-fourth of individuals in the world[14], reinforcing its importance in the etiology of HCC[15]. In 2019, 36300 new cases of HCC and 34700 HCC-related deaths were attributed to NAFLD[16]. The increasing burden of NAFLD will probably lead to a growth in the age-standardized incidence rate of NAFLD-related liver cancer, with an estimated average percentage change of 2.12 between 2018 and 2030[13]. The growing importance of NAFLD as a cause of HCC becomes apparent when two cohorts from South America, a continent with a high prevalence of NAFLD, are compared. While from 2005 to 2015, 9% of HCC cases were attributed to NAFLD[17], 34% of cases were associated with NAFLD from 2019 to 2020[18].

Most cases of HCC develop in cirrhotic livers. Nevertheless, it is noteworthy that HCC may also occur in NAFLD without cirrhosis[15,19-21]. Aside from cirrhosis, diabetes and other metabolic traits, older age, male sex, alcohol consumption and tobacco smoking also seem to be risk factors for developing HCC in patients with NAFLD[10,22-24]. In cirrhosis associated with NAFLD, the annual incidence of HCC is reported as 0.5% and 2.6%[25,26].

In a large retrospective cohort study of European primary care databases, including 136703 patients with NAFLD and matched controls, the incidence rate of HCC was 0.3 per 1000 person-years among individuals with NAFLD, which was significantly higher than among controls, with a hazard ratio of 3.51. The risk of developing HCC was higher according to the Fibrosis-4 (FIB-4) score, which might reflect the odds of having cirrhosis[22].

In another retrospective cohort study performed using a large American administrative database, including 296707 individuals with NAFLD and an equal number of matched controls, HCC was diagnosed in 490 patients with NAFLD and 55 controls. This translated into an annual incidence rate of HCC of 0.21 cases per 1000 person-years among individuals with NAFLD, which was significantly higher than among controls (0.02 cases per 1000 person-years). In a subgroup analysis, the annual incidence rate of HCC was 10.6 per 1000 person-years among individuals with cirrhosis, 0.08 per 1000 person-years among those with NAFLD without cirrhosis and 0.02 per 1000 person-years among controls[27]. Nonetheless, the study had substantial methodological limitations, especially regarding misclassification risks and lack of database granularity. Therefore, its results should be interpreted with caution.

Regarding non-cirrhotic HCC, a meta-analysis has demonstrated that around 38% of NAFLD-related HCCs are diagnosed in individuals without cirrhosis[9]. However, it should be emphasized that the risk of liver cancer is substantially higher in patients with NAFLD and cirrhosis when compared to those without cirrhosis. A recent meta-analysis found an incidence of 3.78 *vs* 0.03/100 person-years in patients with non-cirrhotic NAFLD[28].

Table 1 shows the studies that evaluated the incidence/prevalence of HCC and risk factors in patients with NAFLD without cirrhosis.

PATHOPHYSIOLOGY OF NAFLD-RELATED HCC

The pathophysiology and etiology of NASH progression to HCC are not entirely known, and many mechanisms have been proposed. Neoplastic transformation of NAFLD is driven by metabolic imbalance, lipotoxicity consequent to hepatocyte lipid overload, oxidative stress and immunological aspects, whereas many other factors such as genetic markers, gut dysbiosis and alcohol or tobacco abuse may interact as risk modifiers[29].

Genetic factors

Three main single-nucleotide polymorphisms (SNPs) have been described as associated with a higher risk of steatosis, fibrosis and even HCC, Patatin-like phospholipase domain-containing 3 (*PNPLA3*), membrane-bound o-acyltransferase domain-containing 7 (*MBOAT7*), and transmembrane 6 superfamily member 2 (*TM6SF2*) genes[30].

The variant in the *PNPLA3* gene is the strongest genetic variant predisposing from fatty liver to HCC, and its frequency ranges from 17% to 49% according to ethnicity and the geographic distribution of NAFLD[31]. This variant codifies adiponutrin, a protein responsible for the export of lipids from the liver. The substitution of a single nucleotide (from isoleucine to methionine – I148M) modifies the function of adiponutrin, leading to the accumulation of triglycerides, retinyl esters in lipid droplets in both hepatocytes and hepatic stellate cells, leading to fibrogenesis and tumorigenesis. Patients with at least one G allele, primarily those with GG homozygosis, have a higher risk of developing steatosis, fibrosis and HCC[32]. A subgroup analysis from a systematic review involving 9915 patients showed an association between the *PNPLA3* rs738409 SNP and HCC among patients with NASH or alcohol-related cirrhosis with an odds ratio of 1.67 and a 95% confidence interval of 1.27-2.21, but not among patients with cirrhosis of other etiologies[33].

Studies investigating *MBOAT7* association with HCC are scarce. In a cohort of 765 Italian patients with NAFLD, especially those without advanced fibrosis, the *MBOAT7* rs641738 variant was strongly associated with HCC. On the other hand, it showed no association with HCC in a validation cohort of 358 patients with NAFLD without cirrhosis in the United Kingdom[34].

TM6SF2 polymorphism is also associated with increased liver fat content in NASH, advanced hepatic fibrosis and cirrhosis. *TM6SF2* variants have a moderate to significant effect on the risk of NAFLD. Additionally, the *E167K* allele has an allelic odds ratio of 1.82 for steatosis[30]. Whether or not the variant is associated with an increased risk of NAFLD-related fibrosis and HCC remains to be determined.

Recently, the odd-skipped related transcription factor 1 (*Osr1*) has been reported as a novel tumor suppressor gene, as well as a potential prognostic biomarker in gastric cancer. Some authors suggest that *Osr1* plays an essential role in regulating cell survival, cell inflammation, and macrophage migration in the liver. Accordingly, *Osr1* was identified as a novel repressor gene in the progression of NAFLD/NASH[35]. So far, the role of *Osr1* in the progression of NAFLD towards HCC development is not established.

Human telomerase reverse transcriptase (*hTERT*) mutations are associated with familial liver diseases. Telomere length and germline *hTERT* mutations were evaluated to determine their association

Table 1 Studies that included the incidence/prevalence and risk factors for hepatocellular carcinoma in non-alcoholic fatty liver disease without cirrhosis

Ref.	Study design	Aim	Number of patients	Results and conclusion
Mohamad *et al*[5], 2016	Retrospective	To characterize patients with NAFLD and HCC comparing cirrhotic *vs* non-cirrhotic patients	All patients with NAFLD and HCC between 2003-2012 (n = 83)	36 (43.4%) NAFLD HCC non-cirrhotic *vs* 47 (56.6%) NAFLD HCC cirrhotic patients. HCC patients without cirrhosis are more likely to present at an older age with larger tumor and higher rates of tumor recurrence
Piscaglia *et al*[6], 2016	Multicenter observational prospective	To assess the clinical features of patients with NAFLD-related HCC and to compare to those with HCV related HCC	N = 756 (145 NAFLD *vs* 611 HCV)	Cirrhosis was present in about 50% of NAFLD-HCC patients, in contrast to the near totality of HCV-HCC. Survival was significantly shorter in patients with NAFLD-HCC than in those with HCV-HCC (25.5 mo *vs* 33.7 mo)
Stine *et al*[9], 2018	Systematic review with meta-analysis	To compare the prevalence of NAFLD-related HCC to other chronic liver diseases	19 studies (n = 168571)	The prevalence of NAFLD-related HCC in patients with NASH without cirrhosis is approximately 38% compared with 14% for other liver diseases
Tobari *et al*[24], 2020	Prospective	To evaluate the characteristics of HCC in non-cirrhotic NAFLD	48 non-cirrhotic HCC *vs* 71 cirrhotic HCC patients	In patients with non-cirrhotic NAFLD, important risk factors for HCC were male gender, alcohol consumption, and the FIB-4 index. HCC recurrence and survival were only influenced by the tumor stage
Kanwal *et al*[27], 2018	Retrospective	To estimate the risk of incident HCC among patients with NAFLD	296707 NAFLD *vs* 296707 matched controls	NAFLD individuals with cirrhosis had the highest annual incidence of HCC. 20% of NAFLD patients with HCC had no evidence of cirrhosis. The absolute risk of HCC in patients without cirrhosis is too low to recommend HCC surveillance
Orci *et al*[28], 2022	Systematic review with meta-analysis	Evaluate the pooled HCC incidence in patients with NAFLD at distinct severity stages	18 studies (470404 individuals)	Evidence documenting the risk in patients with NASH or simple steatosis is limited, but the incidence of HCC in these populations may lie below thresholds used to recommend a screening (0.03 per 100 person-years)
Donati *et al*[34], 2017	Sectional	To evaluate whether the *MBOAT7* rs641738 risk T allele predisposes to HCC in NAFLD patients stratified by the presence of severe fibrosis	765 Italian NAFLD patients	The *MBOAT7* rs641738 T allele is associated with reduced *MBOAT7* expression and may predispose to HCC in patients without cirrhosis
Demirtaş *et al*[71], 2021	Retrospective	To investigate the characteristics and survival course of non-cirrhotic individuals with HCC	N = 384 HCC; 43 (11.2%) without cirrhosis; 10 (23%) with NAFLD	HCC in non-cirrhotic liver is diagnosed at more advanced stage and with larger tumor size. The overall survival is shorter in HCC without cirrhosis, due to late recognition

NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; *MBOAT7*: Membrane-bound o-acyltransferase domain-containing 7.

with NAFLD-HCC. The authors observed an association between shorter peripheral blood telomeres and NAFLD-HCC development and found that rare germline mutations in *hTERT* predispose NAFLD progression to HCC, potentially assisting the identification of high-risk individuals[36].

Metabolic imbalance

Insulin resistance (IR) is the key pathogenic event associated with the development of hepatic steatosis and is also related to the development of HCC[37].

Hyperinsulinemia can promote the synthesis and activity of insulin-like growth factor-1, inhibiting cell proliferation and apoptosis[38], which increases the risk of hepatocellular carcinogenesis. Hyperglycemia provides a substrate for energy metabolism in tumor cells and leads to a glycosylation reaction activating the inflammatory signaling cascades and generating reactive oxygen species (ROS) to induce HCC development[39]. IR may also directly accelerate hepatocarcinogenesis by stimulating hepatic neovascularization[40].

These events affect cell growth by inducing the transcription of the protooncogenes, so fibrosis and carcinogenesis are promoted in the liver. Additionally, hyperinsulinemia increases hepatic lipid accumulation and leads to oxidative stress due to the increased beta-oxidation of free fatty acids and the formation of ROS. There is positive feedback between oxidative stress in mitochondria and endoplasmic reticulum (ER) through ER stress, further contributing to cell injury and carcinogenesis in NASH. In contrast to insulin-mediated apoptosis inhibition, hepatic lipotoxicity activates proapoptotic cell signals. Another recently discovered mechanism involves the association between lipolysis and autophagy, with conflicting evidence due to its double-natured, divergent role in NASH-associated HCC[41].

Lipotoxicity

Lipotoxicity is the dysregulation of intracellular lipid components resulting in the accumulation of

harmful lipids, which are associated with cellular damage and death[42]. Lipotoxicity causes cellular damage as lipids alter the biology and function of intracellular organelles, such as the ER and mitochondria. Also, a direct modification of intracellular signaling pathways may occur, deregulating the metabolic and inflammatory pathways[43].

The ER is an intracellular organelle that engages in many critical cellular processes, including folding membranes and secreted proteins, synthesizing lipids and sterols, and storing free calcium. Disturbance of any of these processes results in stress on the ER and interrupts the protein folding process. When ER stress cannot be restored, the apoptotic pathway is stimulated, leading to cell death to eliminate the stressed cells[44].

ER stress is linked to the development and progression of liver inflammation. Because it is a crucial mediator of liver inflammation, the immunoglobulin protein promotes the inflammatory response associated with NASH[45]. ER stress has been identified as a mediator of NAFLD-promoted HCC *in vitro*. Also, enhanced ER stress increases tumor necrosis factor production by macrophages, leading to tumor formation[46].

Oxidative stress

Oxidative stress results from an imbalance between the excessive formation of prooxidants (ROS and/or reactive nitrogen species) and limited antioxidant defenses, leading to cell death and tissue damage[47].

In NAFLD, there are some mechanisms for producing mitochondrial ROS. Thus, mitochondrial dysfunction and ROS production are exacerbated. In this context, some hepatocytes may develop adaptive cell survival and proliferation mechanisms that promote precancerous transformation and/or tumor growth[48].

Immunological aspects

During the progression of NAFLD from steatosis to NASH and more advanced stages of NASH with liver fibrosis, the immune system plays an important role. There are inflammation triggers within hepatic (lipid overload, lipotoxicity, oxidative stress) and extra-hepatic systems (gut-liver axis, adipose tissue, skeletal muscle), resulting in unique immune-mediated pathomechanisms in NAFLD[49].

Immune cells play a role in hepatocarcinogenesis through processes that are independent of fibrosis. Hepatocyte damage promotes neutrophil infiltration in the liver, resulting in DNA damage to other hepatocytes and promoting HCC development without fibrosis. Furthermore, lymphoid aggregates are often present in the setting of chronic inflammation. Additionally, the selective loss of CD4+ T lymphocytes occurs, which was shown to be critical for the progression of HCC[41].

Although immunological response can promote HCC, the immune system also plays an important role in suppressing tumor growth through immunosurveillance. Furthermore, HCC actively promotes tumor tolerance by inducing immunosuppression, and the fibrotic microenvironment leads to the overproduction of transforming growth factor beta, a potent immunosuppressant, thereby promoting disease progression[41].

Microbiota

Increased gut permeability and altered microbiome composition are associated with NAFLD and its disease severity, contributing to hepatocarcinogenesis[50].

The gut microbiota has been described as a cofactor in liver disease progression and in the development of HCC through the interaction with immune compartments *via* the gut–liver axis. Dysbiosis characterizes the microbiota of patients with NAFLD-cirrhosis, with compositional and functional shifts occurring with HCC development. It has been suggested that the gut microbiota in NAFLD-HCC is characterized by a distinctive microbiome/metabolomic profile and can modulate the peripheral immune response[50]. Human metagenomic data support an emerging core microbiome signature that characterizes NAFLD-cirrhosis, with increased *Ruminococcus gnavus*, *Clostridium bolteae*, *Streptococcus parasanguinis*, and *Klebsiella pneumoniae*, and a reduced number of beneficial species, including *Faecalibacterium prausnitzii*, *Alistipes putredinis*, and *Eubacterium eligens*. Furthermore, *Veillonella parvula* and *Bacteroides caecimuris* are also identified to distinguish NAFLD-HCC from NAFLD-cirrhosis. In agreement with these findings, rRNA analyses of patients with NAFLD-HCC have detected enrichment in *Bacteroides* and *Ruminococcaceae*, which correlated with several systemic inflammatory and immune markers[51]. Ren *et al*[52] also observed a decrease in butyrate-producing bacterial families, namely *Ruminococcus*, *Oscillibacter*, *Faecalibacterium*, *Clostridium IV*, and *Coprococcus* in patients with HCC.

Increased intestinal permeability, intestinal bacterial overgrowth and elevated serum endotoxin have been reported in NAFLD and NAFLD-HCC[53]. Endotoxemia-induced toll-like receptor 2 induction leads to cyclooxygenase-2 (COX2) mediated prostaglandin E (PGE) production, which suppresses antitumor immunity by inhibiting antitumor cytokine production from liver immune cells leading to HCC progression in a mouse model. In human non-cirrhotic NAFLD-related HCC, COX2 overexpression and excess PGE production are detected. Although these findings suggest that hepatocellular inflammation may be secondary to altered intestinal permeability and translocation of either intact bacteria or microbial cell components into the circulation, the causal link between them is not entirely

clarified[53,54].

Other factors

Many factors have been associated with the potential to increase the risk of HCC in NAFLD, such as male gender, older age, ethnicity, presence of type 2 DM, obesity, any degree of alcohol consumption and smoking[27,55].

Among these, risk factors for NAFLD-related HCC, which have long been recognized, are male sex, older age and Latino ethnicity[56]. Kanwal *et al*[27] described in a large cohort study involving 296707 patients with NAFLD that age above 65 years was an independent risk factor for HCC. It was more often identified in men and was higher in Hispanic individuals compared to white (0.21 per 1000 patient-years) and African American individuals (0.12 per 1000 patient-years)[27].

Clinical variables such as diagnosis of type 2 DM and obesity are also significant risk factors among patients with NAFLD. They can act independently or jointly with NAFLD to increase the risk of HCC development[56]. Type 2 DM doubled the risk of developing this outcome[19]. DM is a recognized risk factor for HCC regardless of the etiology of liver disease, and some authors suggest that DM has the strongest association with HCC[57], being related to the duration of DM and adequate glycemic control [58]. On the other hand, it is unclear if the correlation between DM and HCC in patients without cirrhosis applies, as a recent study evaluating the differences between cirrhotic and non-cirrhotic HCC in NAFLD found an inverse association between DM and HCC in the non-cirrhotic group, emphasizing that non-cirrhotic HCC tended to occur in older patients and those with a lower body mass index[59].

Obese patients with cirrhosis were 47 times more likely to have HCC than persons without liver disease, and there is strong evidence that obesity impacts HCC development and promotes an increase in mortality, especially in those with early age onset and the presence of visceral fat[58].

Obesity is a well-known risk factor for many cancers but is significantly linked to liver cancer[60]. A study from the Mayo Clinic has shown that the diagnosis of type 2 DM increased the risk of HCC by fourfold. Therefore, it is recommended that type 2 DM in every individual with NAFLD should be investigated due to its association with more advanced disease and increased risk of HCC[61].

Alcohol consumption is independently associated with a higher risk of HCC in individuals with NAFLD[62]. Some studies suggest that the increased risk would apply only to those with heavy alcohol use (*e.g.*, > 50 g/d or ≥ 3 drinks/d or ≥ 7 drinks/d), better supporting the recent definition of MAFLD instead of NAFLD. The additive effect of alcohol in those with NAFLD might explain the increase of HCC in this specific group[63].

The study by Ascha *et al*[64] suggested that any degree of alcohol consumption may increase the risk of HCC occurrence in patients who, by the classic definition, do not have a significant intake. The deleterious effects of continuous and excessive ethanol intake on the liver are well established; however, there is uncertainty regarding the impact of mild to moderate ethanol consumption[65].

In the same way, elevated alanine aminotransferase has been proposed as an independent factor associated with an increased HCC risk[65].

Environmental factors such as tobacco smoking are associated with insulin resistance, the development of NAFLD and liver cancer. Current and former smoking is associated with a 70% and 40% increased risk of liver cancer, respectively[66]. Similarly, in a meta-analysis of 81 studies, the pooled odds ratios for HCC development were 1.55 in current smokers *vs* 1.39 in former smokers[67]. Currently, there is no specific data on the risk of smoking in NAFLD-related HCC.

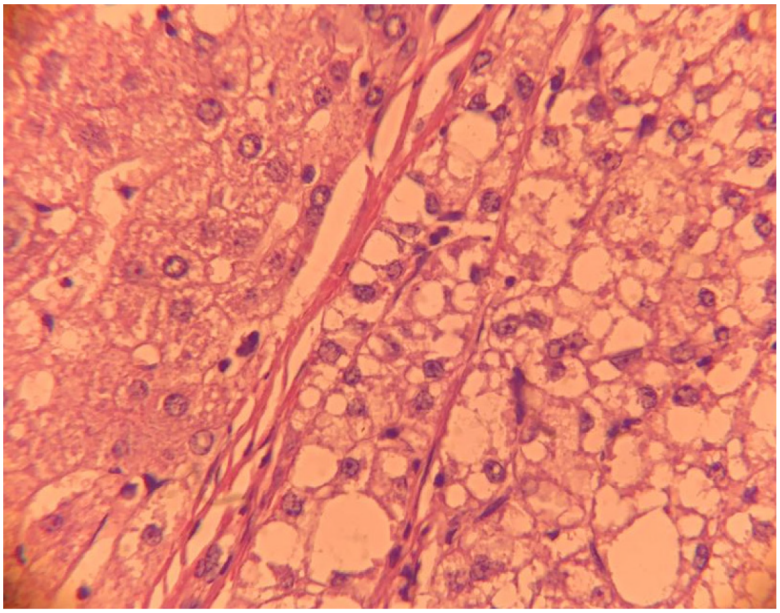
Many studies[22,27,54,57,64,65,68] have assessed the risk of HCC or other liver complications in patients with non-cirrhotic NAFLD, but they have many limitations. Most of them were retrospective and heterogeneous in terms of the inclusion criteria; did not have data on liver fibrosis stages; or had a short follow-up to assess complex outcomes such as HCC or complications of cirrhosis. In addition, most of them had relatively few cases of HCC diagnosed.

HISTOPATHOLOGICAL ASPECTS OF NAFLD-RELATED HCC

Patients with NAFLD and HCC without cirrhosis have larger tumors, but more often, they have well-differentiated tumors and a single nodule compared to those with cirrhosis[59,69,70]. On the other hand, due to late diagnosis, some cases have a higher rate of vascular invasion and extra-hepatic metastasis [71]. Frequently, the nontumor liver has significant steatosis and histological findings of steatohepatitis [72] (Figure 1).

Paradis *et al*[69], studying patients with HCC and metabolic risk factors, demonstrated that the neoplasia in 5 of 31 patients with NASH without cirrhosis developed on a preexisting liver cell adenoma.

Approximately 90% of HCCs are the conventional subtype, but patients with NAFLD with or without cirrhosis or patients with metabolic risks can present a histological subtype of HCC identified as a steatohepatitis-related variant[73]. Macroscopically, the nodule is golden-yellow in color and slightly firm because of steatosis and fibrosis[74].



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Figure 1 Histopathological aspect of a non-alcoholic fatty liver disease-related hepatocellular carcinoma in a non-cirrhotic liver. On the left side: Non-cirrhotic liver with steatosis. On the right side: Hepatocellular carcinoma, steatohepatitis variant with steatosis, hepatocellular ballooning and macrotrabecular arrangement.

The histologic features of this subtype are macrovesicular steatosis, ballooning malignant hepatocytes, lymphocytic inflammation, Mallory Denk bodies and pericellular fibrosis, often with a "chicken-wire pattern"[75]. More frequently, neoplasia has a trabecular arrangement and minimal mitotic activity[74,75].

Regarding immunohistochemistry, a study evaluating 62 cases demonstrated a similarity between steatohepatitis HCC in non-cirrhotic livers and inflammatory liver cell adenomas, demonstrating a higher expression of C-reactive protein and serum amyloid A[70].

These tumors also have distinct molecular features: They frequently showed IL-6/JAK/STAT activation and less often Wnt/β catenin/CTNNB1 and P53 pathway alterations[76].

CURRENT RECOMMENDATIONS ON SURVEILLANCE FOR HCC IN NAFLD

HCC in the setting of NASH is known to occur even in the absence of liver cirrhosis, an event previously mainly related to hepatitis B virus infection. Thus, knowing whom to screen for HCC and which patient population deserves surveillance is essential.

The objective of screening and surveillance in patients with cirrhosis is to reduce mortality, as this patient population will develop HCC. Cost-effectiveness studies suggest that an incidence of 1.5% per year or more would warrant HCC surveillance in cirrhotic patients, regardless of etiology[23,77]. Recent analysis has confirmed the importance of surveillance in patients with cirrhosis, resulting in longer survival[78]. In Brazil, when we performed screening in a population of more than 500 patients with cirrhosis, the prevalence of HCC was around 5%[79]. Likewise, when we followed a cohort of 450 patients with cirrhosis, the estimated cumulative incidence of HCC was 2.6% in the first year, 15.4% in the fifth year and 28.8% in the tenth year, demonstrating the relevance of carrying out a surveillance program[80].

NAFLD, with or without NASH, is a hepatic manifestation of metabolic syndrome and predisposes to HCC in cirrhotic and non-cirrhotic patients. Despite the high prevalence of NAFLD in the general population, as previously mentioned, it is believed that the incidence of HCC in these patients with non-advanced disease is not sufficiently high for a universal surveillance program to be proposed. In a systematic review, considering only studies that include patients with or without cirrhosis, the incidence of HCC in NAFLD patients with cirrhosis was 15% at 10 years, while the incidence in NAFLD patients without cirrhosis was 2.7% and 23 per 100000 person-years[81]. Given the lowest risk of HCC in non-cirrhotic livers (approximately 0.1 to 0.8 per 1000 patient-years), the development of cost-effective HCC surveillance strategies to identify high-risk NAFLD patients without cirrhosis are needed[58].

Although type 2 DM and obesity have been implicated as independent risk factors for HCC, studies establishing a clear link with HCC in non-cirrhotic livers are scarce[82]. Therefore, it becomes essential to assess the benefits of predictive models based on clinical data to identify patients with HCC in the

population of NAFLD patients without cirrhosis.

Some authors use different tools to stratify patients according to the risk of developing HCC. Thus, FIB-4 was evaluated in European databases, including more than 18 million individuals. When the NAFLD group was classified according to the FIB-4 score, it was possible to identify which patients were at greater risk. When compared to individuals with a FIB-4 score < 1.30, those with a score between 1.30 and 2.67 had a risk ratio for HCC of 3.74, and those with a score > 2.67 had a risk ratio for HCC of 25.2[22]. Although not accepted by all[27], it is possible that the FIB-4 score can be used in selected patients for surveillance.

In a European longitudinal study, Younes *et al*[83] applied various scores (NAFLD fibrosis score - NFS, FIB-4, BARD, APRI) and the Hepamet fibrosis score to predict HCC in 1173 patients with NAFLD (75% non-cirrhotic). These patients were followed for a mean period of 81 mo, with 17 patients (1.5%) developing HCC. The NFS performed significantly better than the other non-invasive scores (C-index: 0.901 ± 0.0302; AUROC = 0.889 ± 0.048)[83].

The latest European Association for the Study of the Liver guideline recommends surveillance in patients with metabolic syndrome or NASH in the presence of significant fibrosis on histology or elastography. However, it is noted that the role of surveillance for NAFLD patients without cirrhosis is unclear[84].

Recently, at a meeting of experts, an evidence-based review was performed addressing the risk of HCC in patients with NAFLD. This review concluded that NAFLD patients with evidence of advanced fibrosis, even when suggested by non-invasive markers, should be considered for HCC screening. Thus, the need for surveillance would be indicated when there is an agreement between two non-invasive tests with different methodologies (FIB-4 and elastography, for example). These results were endorsed by the American Gastroenterological Association[85].

NOVEL PERSPECTIVES IN SURVEILLANCE FOR HCC IN NAFLD

The most validated predictive factor for HCC development in NAFLD is the presence of advanced fibrosis. However, many other factors may be considered to identify those at high risk for liver cancer, even though we still do not have enough evidence to change HCC surveillance strategies in NAFLD[86].

In addition to surveillance based on imaging and serological methods, mainly ultrasound and alpha-fetoprotein, there are no scores or predictive models with enough strength to use in the daily surveillance of NAFLD-related HCC. The development of novel tools might help risk stratification and accurately identify high-risk patients, even those without cirrhosis, leading to individualized surveillance strategies.

In future studies, some of these clinical scores should be combined with genetic risk factors for risk stratification of patients with NAFLD, since the genetic markers currently available still have limitations. As noted, different genetic polymorphisms have varying effects on HCC risks; the 17-β hydroxysteroid dehydrogenase 13-HSD17B13, for example, has protective effects, while others such as the *PNPLA3* (variant I148M) increase HCC risk[58].

The combination of genetic polymorphisms to determine a genetic risk score has shown a low accuracy with a sensitivity of 43% and specificity of 79% in the prediction of HCC with an AUROC of only 0.65[85]. Moreover, the genetic polymorphisms are not ready to be used in clinical routine due to high cost and low availability. Another large study by Bianco *et al*[87] investigated the polygenic risk score (PRS) in a German and an Italian cohort with NAFLD compared to the general population regarding the development of HCC. The polygenic risk score (PRS) was composed of TM6SF2-GCKR-MBOAT7 combined in hepatic fat PRS (PRSHFC), further adjusted for HSD17B13 (PRS-5). This study showed a strong association between hepatic fat and HCC. The PRS improved the accuracy of HCC detection and may help stratify HCC risk in individuals with dysmetabolism, including those without severe liver fibrosis[87].

Also, multiple new panels, including biomarkers such as multiprotein-based and circulating tumor-derived DNA-based ("liquid biopsy") panels[88], as well as abbreviated magnetic resonance imaging protocols and other imaging-based protocols, are currently under investigation as potential screening tests. Studies investigating the accuracy of liquid biopsies are ongoing. Liquid biopsy strategies for sampling tumor products in the bloodstream include substances such as circulating tumor cells (CTCs), circulating tumor DNA (ct-DNA) and extracellular vesicles (EVs)[88]. CTCs include cells released from primary or metastatic tumor sites, CT-DNA consists of DNA from cellular necrosis or apoptosis, and EVs are cell membrane-derived particles such as apoptotic bodies, micro-vesicles and exosomes, containing molecular cargoes specific to the origin cell with an essential role in cell-to-cell communication[88]. Data from a systematic review with 67 studies evaluated liquid biopsy techniques for early-stage HCC detection, including studies evaluating CTCs, ct-DNA and EVs. They have shown good accuracy for HCC detection, with higher accuracy than alpha-fetoprotein (AFP) for distinguishing patients with HCC from controls and the capacity to identify AFP-negative HCC patients. In this study, combinations with AFP were superior to AFP alone[62]. When included in a panel, a liquid biopsy was also associated with poorer survival (EVs and ct-DNA)[89] and with tumor progression.

Some blood-based biomarkers, such as lectin-bound AFP (AFP-L3) and des-gamma carboxyprothrombin (DCP), have been proposed for detecting HCC in some regions like Japan and are under investigation in other countries. Moreover, there is an increased interest in early detection panels using multiple combined biomarkers. The best example is GALAD, which combines demographic and clinical variables with blood-based biomarkers such as gender, age, AFP, AFP-L3, and DCP[90]. In a multinational case-control study, its sensitivity was 60%–80% for detecting early-stage HCC[90]. The GALAD panel was recently evaluated in a case-control study of 125 patients with NAFLD. It showed a similar diagnostic performance at a cut-off of -0.63, with a sensitivity and specificity of 68% and 95%, respectively, for early-stage HCC[91]. Interestingly, in the prospective study arm, the GALAD score identified patients who developed HCC as early as 1.5 years before their diagnosis[91]. However, although it is a promising tool, it is not yet available for clinical use since it still needs to be validated in phase III and IV studies.

After basic serological tests, elastographic techniques are the cornerstone for NAFLD's non-invasive staging of liver fibrosis. Vibration-controlled transient elastography (VCTE) can also assess steatosis through the controlled attenuation parameter and is considered the point of care method among elastography-related techniques[40]. 2D-Shear wave elastography and point-shear wave elastography have the additional capacity to evaluate the macroscopic aspect of the liver and identify nodular lesions as patients with NAFLD-related cirrhosis should have an ultrasound every six months to screen for HCC. Thus, the elastography evaluation and the evaluation of liver lesions have been studied as additional methods for HCC surveillance[92]. A recent study in type 2 DM individuals with NAFLD who had VCTE at baseline and were followed for 50 mo has shown that those with liver stiffness > 13 kPa had a higher incidence of liver decompensation and HCC[93].

Boursier *et al*[94] evaluated the prognostic significance of liver stiffness in NAFLD. They proposed defining a new fibrosis classification stage based on liver stiffness by VCTE categorized in seven different classes of liver fibrosis: LSM1 (2.0 to 4.6 kPa), LSM2 (4.6 to 6.1 kPa), LSM3 (6.1 to 8.8 kPa), LSM4 (8.8 to 12.0 kPa), LSM5 (12.0 to 18.0 kPa), LSM6 (18.0 to 38.6 kPa) and LSM7 (greater than 38.6 kPa to 75 kPa). In this study, overall survival decreased as liver stiffness increased. For instance, overall survival for LSM1 in ten years was close to 100%, whereas, for LSM7, it was near 30%. The authors evaluated liver-related deaths in this study, not specifically HCC[94]. As a reflection, based on the data presented, we could suggest performing elastography in patients with NAFLD, and, when a greater liver stiffness is evidenced, they would be selected to join a screening and surveillance program.

CONCLUSION

It is currently challenging to propose general recommendations for screening patients with NAFLD without cirrhosis, and each patient should be evaluated on a case-by-case basis based on the profile of specific risk factors identified. For HCC screening in NAFLD, a valid precision-based screening is needed.

Currently, when evaluating this population of patients, we believe that the use of non-invasive methods can guide the selection of patients who will undergo a screening and surveillance program. So far, ultrasound with or without AFP is still the screening method of choice and should be used for all NAFLD patients with advanced fibrosis. In the future, it is possible that new technologies and liquid biopsy methods might add precision in screening these large populations, including those without cirrhosis.

FOOTNOTES

Author contributions: Tovo CV and de Mattos AA conceptualized the study; all authors contributed equally to drafting the article and making critical revisions related to important intellectual content of the manuscript; and all authors approved the final version of the article to be published.

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Country/Territory of origin: Brazil

ORCID number: Cristiane Valle Tovo 0000-0002-7932-5937; Angelo Zambam de Mattos 0000-0002-3063-0199; Gabriela Perdomo Coral 0000-0003-4318-2871; Giovana D P Sartori 0000-0002-2701-3499; Livia Villela Nogueira 0000-0002-5429-

7132; Gustavo Tovo Both 0000-0003-4054-1242; Cristiane A Villela-Nogueira 0000-0003-1355-2368; Angelo A de Mattos 0000-0003-2417-9765.

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MINIREVIEWS

Secondary bile acids and the biliary epithelia: The good and the bad

Ilaria Lenci, Martina Milana, Alessandro Signorello, Giuseppe Grassi, Leonardo Baiocchi

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Ilaria Lenci, Martina Milana, Alessandro Signorello, Giuseppe Grassi, Leonardo Baiocchi, Hepatology Unit, Policlinico Tor Vergata, Rome 00133, Italy

Corresponding author: Leonardo Baiocchi, MD, PhD, Associate Professor, Hepatology Unit, Policlinico Tor Vergata, Via Montpellier 1, Rome 00133, Italy. baiocchi@uniroma2.it

Abstract

The biliary tract has been considered for several decades a passive system just leading the hepatic bile to the intestine. Nowadays several researches demonstrated an important role of biliary epithelia (*i.e.* cholangiocytes) in bile formation. The study of biliary processes therefore maintains a continuous interest since the possible important implications regarding chronic cholestatic human diseases, such as primary biliary cholangitis or primary sclerosing cholangitis. Bile acids (BAs), produced by the liver, are the most represented organic molecules in bile. The physiologic importance of BAs was initially attributed to their behavior as natural detergents but several studies now demonstrate they are also important signaling molecules. In this minireview the effect of BAs on the biliary epithelia are reported focusing in particular on secondary (deriving by bacterial manipulation of primary molecules) ones. This class of BAs is demonstrated to have relevant biological effects, ranging from toxic to therapeutic ones. In this family ursodeoxycholic and lithocholic acid present the most interesting features. The molecular mechanisms linking ursodeoxycholic acid to its beneficial effects on the biliary tract are discussed in details as well as data on the processes leading to lithocholic damage. These findings suggest that expansion of research in the field of BAs/cholangiocytes interaction may increase our understanding of cholestatic diseases and should be helpful in designing more effective therapies for biliary disorders.

Key Words: Cholangiocytes; Biliary secretion; Cholestasis; Bile acids; Secondary bile acids; Ursodeoxycholic acid; Lithocholic acid

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Core Tip: The biliary epithelia present important physiologic activities that are of interest with regard to chronic cholestatic liver diseases. Secondary bile acids (BAs) are derived by bacterial manipulation of the primary BAs produced by the liver. This review summarizes the most important recent findings with regard to secondary BAs interaction with biliary epithelia.

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INTRODUCTION

The biliary system is composed of a delicate structure of anastomosing ducts, leading the bile from the liver toward the intestine[1]. While for several years this anatomical apparatus was considered just as an inert route for bile transport, recently several studies have demonstrated that important qualitative/quantitative bile changes occur within the biliary tract. The isolation and characterization of the biliary epithelia (composed of bile duct cells or cholangiocytes) has deepened our understanding of several important molecular process involving the biliary tree, also shedding some light on the mechanisms leading to chronic cholestatic liver diseases.

Bile acids (BAs) are the main organic molecules secreted in bile[2]. Their physiological importance, which in the beginning was identified only with regard to the physicochemical processes leading to micelle formation[3], nowadays has been expanded by the evidence that BAs are also essential signaling molecules[4]. In this minireview, the most important findings involving secondary BAs and biliary epithelia will be reported together with the possible implications of these mechanisms in human liver diseases.

SECONDARY BAS

BAs are synthesized by the liver starting from cholesterol and are the most represented lipidic component in bile[5]. Taurine or glycine conjugation, occurring after synthesis, confers increased water solubility to these molecules in bile. BAs are traditionally classified as primary (produced by the liver) or secondary (derived by primary BAs after bacterial dehydroxylation in the intestine)[6]. In humans, the primary BAs are cholic (CA) and chenodeoxycholic (CDCA) acid, while the most represented secondary ones are deoxycholic (DCA) and lithocholic (LCA) acid. The removal of a hydroxyl group (C-7 position) in general determines reduced water solubility and increased detergency in comparison with primary precursors. The hydrophilic or hydrophobic character of a specific BA has been put in relation with its potential cytotoxicity and damaging effects[7]. In this perspective, secondary BAs are generally regarded as possibly damaging molecules when they reach adequate concentrations since their detergent/destabilizing effect on cell membranes. Being the bile a mixture of different (primary and secondary) BAs, the concept of hydrophilic/hydrophobic balance of the bile (and so the net concentration of secondary BAs) has been related with possible liver injury in some conditions[8]. The most hydrophobic human BA is the monohydrate LCA. Sulfation of this molecule by the liver greatly reduces its intestinal absorption (also enhancing its hydrophilicity and urine elimination) and the consequent damage induced by LCA enterohepatic recirculation[9]. It in fact represents less than 5% of total BAs in human bile[6]. The number of hydroxyl groups, however, is not the only determinant of the specific hydrophilic/hydrophobic character of a specific BA. In fact, another secondary BA, ursodeoxycholic acid (UDCA), despite having an equal number of OH groups (two) in comparison with CDCA and one less than CA, is more hydrophilic in comparison with the latter molecules. This physico-chemical characteristic is related to the fact that, differently from CDCA (3α, 7α), in UDCA, the two hydroxyl groups are not on the same plane (3α, 7β). See Table 1 for a quick reference on hydroxyl group number and position, together with some other features, of the principal BAs found in human bile. On Figure 1 the approximate amount of each individual BA in human bile is reported.

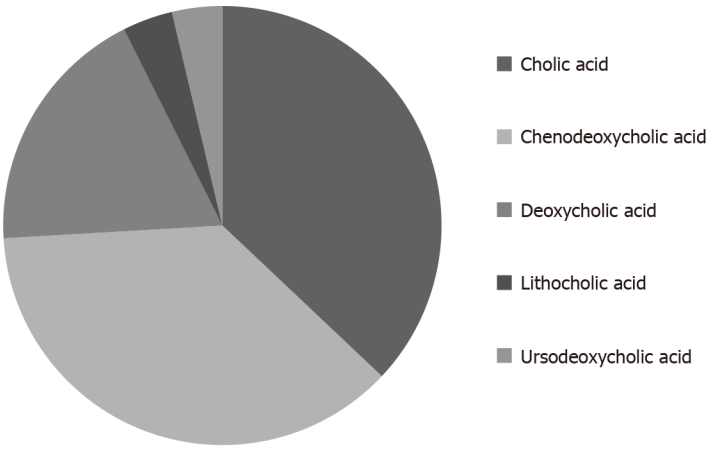
BILIARY EPITHELIA

Together with hepatocytes, cholangiocytes constitute the liver epithelial compartment. These latter cells, lining the intrahepatic and extrahepatic biliary ducts, despite representing less than 10% of liver mass, are able to support nearly 50% of bile volume under stimulation[10]. They in fact contribute almost

Table 1 Some physico-chemical features of the most relevant primary and secondary bile acids in human bile

	Hydroxil groups number and position	Solubility in water (protonated form, μM)[1]	Critical micellar concentration (sodium salt, mM)[1]	Hydrophobicity index (taurine conjugated)[2]
Primary bile acids				
Cholic acid	3 (3, 7, 12)	273	13	0
Chenodeoxycholic acid	2 (3, 7)	27	9	0.46
Secondary bile acids				
Deoxycholic acid	2 (3, 12)	28	10	0.59
Lithocholic acid	1 (3)	0.05	0.9	1
Ursodeoxycolic acid	2 (3, 7)	0.9	19	-0.47

[1]Values assessed in water as reported by Hofmann *et al*[44].
[2]Cholic acid and Lithocholic acid were assumed to have (by definition) a value of 0 and 1 respectively[8].



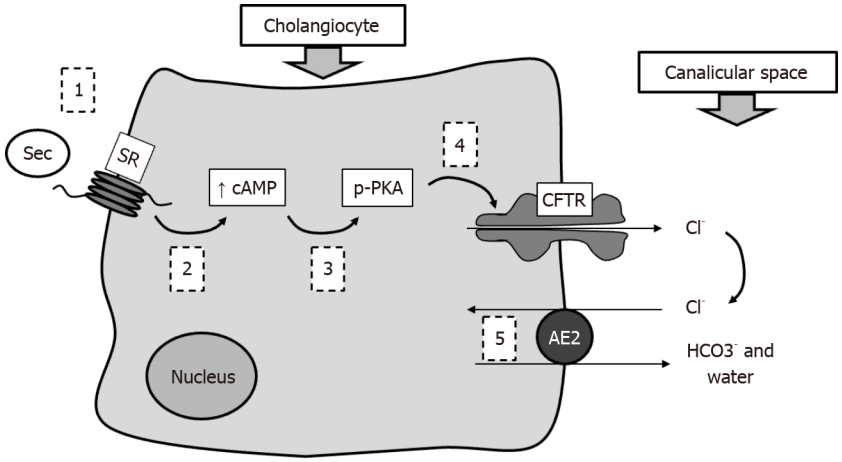
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Figure 1 The relative amount of individual bile acids in human bile is depicted. For each bile acid the extent of conjugation with glicine *vs* taurine is approximately 3 to 1.

exclusively to the so-called BA-independent bile flow. Cholangiocytes are heterogeneous in size and function; the larger ones represent the physiologically functional compartment, while smaller cells (harboring small branches) may replace large ones when the latter are injured[11]. The most studied mechanism of bile duct secretion concerns the interaction between secretin (Sec) and a specific Sec receptor (SR) expressed by cholangiocytes only within the liver. The subsequent downstream molecular mechanisms are characterized by increased intracellular cAMP in bile duct cells, followed by PKC phosphorylation, extrusion of Cl^- by the cystic fibrosis transmembrane regulator and finally its reabsorption and exchange with bicarbonate operated by the Cl^-/HCO_3^- exchanger (AE2)[12]. With this process, a bicarbonate-enriched choleresis is obtained. See Figure 2 for a schematic representation of this mechanism. However, several hormones and neuropeptides (such as somatostatin, histamine, melatonin, gastrin and others) may regulate bile duct cell activity, as these cells have been demonstrated to express the corresponding receptors[13]. BA receptors and transporters are also present on cholangiocytes. They are responsible for important physiological mechanisms.

BAS/BILIARY EPITHELIA INTERACTIONS

The biliary epithelium is constantly exposed to significant concentrations (mM) of BAs. This strict connection is at the basis of important processes under both normal and pathological conditions. As previously stated, BAs are mainly present in bile as glycine or taurine conjugates; however, more than 30 years ago, the possibility that unconjugated BAs may cross the biliary epithelium and recirculate in the liver (the so-called chole-hepatic shunt) was hypothesized, thereby inducing increased choleresis with multiple passages[14]. Later, uptake of BAs by the biliary epithelium was demonstrated by the



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Figure 2 A step by step representation of secretin-induced biliary secretion is depicted. 1: Secretin (Sec) bind to specific Sec receptor on cholangiocytes; 2 and 3: Increased intracellular levels of cAMP stimulate formation of p-PKA; 4: Cystic fibrosis transmembrane regulator is opened determining Cl- efflux; 5: chloride-bicarbonate exchanger (AE2) favors reuptake of Cl- and releases HCO_3- (osmotically recalling water) in the canalicular space. CFTR: Cystic fibrosis transmembrane regulator; Sec: Secretin; SR: Secretin receptor.

identification of the apical sodium-dependent BA transporter (ASBT) on cholangiocytes[15]. ASBT in the same study was demonstrated to be expressed only by cholangiocytes within the liver and to prompt unidirectional BA transport from the apical to the basolateral cellular domain. ASBT is also expressed in the small intestine, actively reabsorbing BAs and having a major role in maintaining the appropriate entero-hepatic recirculation of these molecules. Gene disruption of this transporter, in fact, nearly completely abolished intestinal recovery of BAs[16], even if a reduced proportion of unconjugated protonated BAs is absorbed by passive uptake in the colon[17]. ASBT function in cholangiocytes, however, remains less clear. In one study, it was demonstrated that Sec stimulation of cholangiocytes was able to increase choleresis, also promoting the transfer of ASBT from the plasma membrane to the apical domain and supporting the original concept of the BA cholehepatic shunt[18]. In another study, a relationship between biliary BAs concentration and ASBT expression was found, suggesting a possible regulatory mechanism of this transporter in maintaining an appropriate biliary BAs concentration[19]. At present, ASBT inhibitors are under study to reduce the BA pool in diseases possibly related to its pathological increment, such as primary biliary cholangitis (PBC)[20].

Further information regarding BAs and cholangiocyte molecular interactions came after the identification of the TGR5, specific for BAs[20]. While in the liver the FXR is mainly expressed in the hepatocyte nucleus where it regulates the transcripts for the synthesis of these molecules[21], on the other hand, TGR5 is prevalently found on the cholangiocyte apical domain[22]. Studies on TGR5(-/-) mice showed an effect on body weight, the immune system and glucose homeostasis[23]. With regard to the biliary tree, TGR5 seems to be an important regulator of cell proliferation with the opposite effect when it is activated in ciliated *vs* non-ciliated cells[24]. In fact, activation of TGR5 on cholangiocyte cilia depresses cAMP formation and proliferation while the same signal in non-ciliated cholangiocytes enhances intracellular cAMP and cell growth. The important role of TGR5 as a possible regulator of biliary mass suggests that this receptor is a possible target in human diseases characterized by uncontrolled cholangiocyte growth, such as cholangiocarcinoma[25] or polycystic liver disease[26]. More recently, other BA receptors, such as the S1PR2, have been identified on cholangiocytes[27]. These signals enhance biliary growth upon stimulation with taurocholic acid (TCA), employing an ERK1/2 dependent mechanism. In conclusion, accumulating evidence demonstrates that the role of BAs in bile is not restricted to lipid dissolution. In fact, BAs are also important molecular signaling molecules.

SECONDARY BAS AND THE BILIARY EPITHELIA

As previously reported, secondary BAs originate from manipulation of the original molecules synthesized by the hepatocytes, by intestinal bacteria. However, within this family, molecules with opposite physicochemical and biological characteristics cohabit. The extremities of this class of organic compounds, in terms of heterogeneity, are represented by UDCA and LCA. At the same time, these two BAs seem particularly interesting and relevant with regard to human biliary diseases, as evidenced by several studies.

UDCA (the good one)

UDCA was first detected as primary BA in Chinese black bear bile, and later also identified in human bile as a secondary BA, in small amounts (≤ 3%)[28]. Interest in UDCA was first focused on its therapeutic potential for cholesterol gallstone dissolution[29,30]. However, its clinical efficacy for gallstone treatment is: (1) Limited to small (≤ 1 cm) non-calcified stones; and (2) affected by frequent recurrent disease when UDCA is withdrawn. On the other hand, early studies on gallstone dissolution, conducted in patients with concurrent chronic hepatitis, also demonstrated the capabilities of UDCA in improving liver function[31].

UDCA beneficial effects on biliary epithelia (general)

Some clinical studies specifically underscored the UDCA beneficial effects in diseases targeting biliary cells and causing an impaired biliary secretion (*i.e.* cholestasis), such as PBC[32]. UDCA (oral dose 13 to 15 mg/kg/day) is in fact, nowadays, a first line treatment for this disease[33,34]. Several mechanisms seem responsible for the improved clinical picture when UDCA is employed in biliary cholestasis[35]. First, due to its intrinsic hydrophilicity, UDCA seems able to reduce the cytotoxicity/hydrophobicity of the total BA pool against bile duct cells. Second, increased biliary secretion is observed if UDCA enrichment occurs in bile. Finally, immune-modulatory and antiapoptotic effects have been demonstrated[32]. Moreover, also regarding PBC, impairment of AE2 and consequent inadequate formation of a delicate bicarbonate film in the canalicular biliary space (the so-called bicarbonate umbrella) has been suggested to facilitate biliary damage by protonated BAs. In this setting, UDCA seems to be able to reconstitute adequate bicarbonate secretion, thus mitigating PBC injury[36] and also reducing the endoplasmic reticulum stress and autophagy acting as a chaperone[37]. With regard to the biliary epithelium, experimental studies have elucidated some important mechanisms.

Molecular basis of UDCA beneficial effects

In early research, conducted in the cholestatic model of the bile duct ligated (BDL) rat (a condition inducing a hyperplastic growth of the biliary tree), UDCA feeding was able to attenuate biliary mass proliferation[38]. A subsequent study using the same model (BDL) clarified that both pathologically enhanced proliferative and secretive processes of cholangiocytes were mitigated by UDCA, as demonstrated by reduced H3 histone, protein cellular nuclear antigen (PCNA) and SR gene expression, and decreased Sec-induced choleresis[39]. Decreased proliferation was not related to cholangiocyte apoptosis and was dependent (as was decreased secretion) on PKCα activation. Another molecular aspect characterizing the effects of UDCA was the decreased ASBT cholangiocyte expression leading to reduced intracellular BA influx. These findings were extended in a more complex model combining rat BDL and vagotomy. In fact, when vagotomy was performed in the BDL rat, the consequent lack of cholinergic stimuli impaired the hyperplastic cholangiocyte response to cholestasis and led to apoptosis in bile duct cells[40]. When UDCA was administered in this model, it was able to counterbalance bile duct cell loss and apoptosis by a PKCα/Ca^{2+} dependent mechanism[41].

UDCA effects in animal model of human biliary disease

Further information regarding UDCA and biliary epithelia came from the Mdr2(-/-) mice model. This mouse is not able to transport phospholipids in bile and develops a chronic cholestasis, resembling the human primary sclerosing cholangitis (PSC), with similar scars and strictures within the biliary tree[42]. In Mdr2(-/-) mice UDCA attenuated reactive cholangiocyte proliferation as well as inflammatory and fibrotic processes. These effects were in part related to the inhibition of mast cells, which are activated during experimental and human PSC[43].

LCA (the bad one)

LCA is a monohydrate secondary BA that is known for its particular hydrophobicity, remaining water insoluble in its free form while it presents a very low critical micellar concentration (concentration at which micelles are spontaneously formed) in saline[44]. According to its physico-chemical properties, LCA has longer been known as a cholestatic and injurious agent in animal experiments[45,46] and, in parallel with this, increased levels of this BA have been found in human with chronic liver disease[47].

General mechanisms of LCA-induced cholestasis

Several mechanisms were identified at the basis of LCA-induced cholestasis such as: (1) Impairment of bile secretion (both BAs dependent and independent)[48]; (2) bile salt export pump translocation from apical membrane to cytosol with its consequent reduced activity[49]; (3) changes in apical membrane fluidity and tight junction permeability[50]; and (4) impairment of canalicular contraction[51].

LCA effects on biliary epithelia

With regard to biliary epithelia, a study on LCA feeding in Swiss albino mice evidenced interesting features[52]. After 4 d of a 1% LCA diet, destructive cholangitis characterized by stenosis of biliary ducts, solid crystal precipitation and bile infarcts was observed. Neutrophil infiltration surrounded the

Table 2 Main findings regarding secondary bile acids and biliary epithelia

Model	Administration route	Main results	Main molecular, immunologic findings	Ref.
Ursodeoxycholic acid				
BDL rat	Feeding (both the unconjugated and taurine-conjugated form)	Decreased biliary proliferation and secretion. No apoptosis	Decreased H3-histone. PCNA, SR and ASBT expression. No apoptosis. Increased PKC α expression	[38, 39]
BDL + vagotomy rat	Feeding (both unconjugated and taurine-conjugated form)	Reversal of duct loss and apoptosis induced by vagotomy	PKCα/Ca^{2+} dependent mechanism	[41]
Mdr2(-/-) mice	Feeding	Decreased proliferation, inflammation and fibrosis	Inhibition of mast cells activity	[43]
Lithocolic acid				
Mouse	Feeding	Destructive cholangitis, bile duct stenosis, bilary infarcts	Damage related to direct toxic effect and not to neutrophil infiltration	[52, 53]
In vivo rat and isolated cholagiocytes	Feeding (cholic acid or Lithocholic acid both taurine conjugated)	Similar effect in increasing proliferation and secretion	Effect restricted to large cholangiocytes	[54, 55]
In vivo rat and isolated cholagiocytes	Feeding (cholic acid or Lithocholic acid both taurine conjugated)	Cholangiocytes proliferation	Dependent by PKA-mediated ASBT expression	[56]
Deoxycholic acid				
Human gallbladder cancer (specimens and cell lines)	*In vitro* exposure	Increased concentration associated with inhibition of tumor growth	Reduced miR-92b-3p inhibits PI3K/AKT activity	[60]

ASBT: Apical sodium bile acids transporter; BDL: Bile duct ligated; PCNA: Protein cellular nuclear antigen; SR: Secretin receptor.

small biliary branches and periductal fibroblast activation with collagen deposition was reported. A subsequent study, conducted in the same experimental system, helped to clarify that LCA-related biliary damage was dependent on direct toxicity of this BA and not to the immune response since neutrophil inhibition did not significantly change the pathological picture[53]. With regard to secretive and proliferative cholangiocyte activities, *in vitro* experiments demonstrated that LCA and CA (taurine conjugated) had similar effects in promoting biliary growth and Sec-stimulated bile output[54]. These results were observed with the large cholangiocyte population, which is well-known as the main functional pool in the biliary tree. Similar results were later confirmed in *in vivo* experiments[55]. In fact, TCA or TLCA rat feeding (1% diet, 1-4 wk) both similarly increased biliary mass and enhanced cholangiocyte biliary secretion. Further experiments suggested that the TLCA stimulation of cholangiocytes function (similarly to TCA) was associated with increased ASBT activity and consequently enhanced intracellular (PKCα/Ca^{2+} dependent) BA trafficking[56]. This process, with regard to LCA, due to the changes in Ca^{2+} flux, was also related to impaired gap junction permeability and consequent cholestasis [57].

Other secondary BAs

With regard to other secondary BAs that may play a role in human biliary physio-pathology, DCA is the only one possibly reaching significant concentrations (10%-35% of total BAs pool) in human bile[58]. DCA liver toxicity has been well-established since the early 1990s and, in a study on rat feeding, this was enhanced in comparison to LCA due to its increased intestinal reabsorption and bile enrichment [59]. Despite this and concerning the biliary epithelia, one study has raised interest by showing the suppression of gallbladder cancer growth by DCA, possibly due to interference with miR-92b-3p[60]. This miR in fact would be responsible of the activation PI3K/AKT pathway that is enhanced in several tumors and also represents a target for anticancer treatment[61]. Several other secondary BAs may be found in different species[62]. For instance in rodents the main represented primary BA is β-muricholic acid (β-MCA; 3α, 6β, 7β)[63]. Bacterial manipulation of β-MCA may give origin to different secondary BAs including HDCA (3α, 6α)[64]. HDCA is reported as the strongest regulator of BA-sensitive ion channel (BASIC) that is normally expressed in brain, intestine and cholangiocytes only, within the liver [65]. While the exact physiologic function of cholangiocyte BASIC has not been well established, evidences demonstrate enhanced activity of this channel, with increased trans-epithelial ion transport, after exposure to HDCA[66]. This suggests BASIC as a further possible regulator of biliary secretion. Table 2 summarizes the main findings regarding secondary human BAs and biliary epithelia.

CONCLUSION

BAs are important organic molecules. For several decades, researchers have focused on their physico-chemical characteristics, due to their reported detergent properties. From this perspective, the hydrophilic or hydrophobic character of a BA has been considered in the past as the main determinants of physiologic effect. This preliminary view is clearly challenged nowadays, with many studies demonstrating the important molecular signaling systems activated by BAs, not only in the hepatocytes but also in the biliary epithelium. Artificial manipulation of native BA molecules, moreover, has led to the discovery of new agents, such as obeticholic acid, that may be helpful for human therapy[67]. Given all the above, it is clear that the original classification of BAs as primary and secondary compounds only expresses aspects of their synthesis and not necessarily beneficial or negative physiologic effects. Similarly, the division of secondary BAs as good or bad ones (as reported in this review) is questionable, since this does not adequately recapitulate the multitude of effects (probably discovered just in part at the present stage) these molecules may have. In fact, UDCA (generally supposed as beneficial) has been demonstrated to be detrimental in experimental obstructive cholestasis as it can lead to bile infarcts and should not be administered in this clinical condition[68]. On the other hand, LCA has shown interesting curative properties and anti-tumoral and anti-inflammatory effects on intestinal environment, in some studies[69]. In conclusion, UDCA and LCA clearly represent the extremities of a field in which research may growth and a revision in our present beliefs regarding these secondary BAs remains therefore possible in the near future. With regard to normal human physiology and in practice, however, LCA accumulation is prevented by a detoxification system while UDCA is formed only in trace amounts. However, bile enrichment is possible when BAs are exogenously administered to manipulate the BAs pool for therapeutic purposes.

FOOTNOTES

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Country/Territory of origin: Italy

ORCID number: Ilaria Lenci 0000-0001-5704-9890; Martina Milana 0000-0003-2027-0481; Alessandro Signorello 0000-0002-3831-7244; Giuseppe Grassi 0000-0001-9182-8759; Leonardo Baiocchi 0000-0003-3672-4505.

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MINIREVIEWS

Non-alcoholic fatty liver disease and COVID-19: Harmless companions or disease intensifier?

Christoph G Dietrich, Andreas Geier, Uta Merle

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Christoph G Dietrich, Gastro-Praxis Wiesbaden, Leberzentrum Wiesbaden, Wiesbaden 65189, Germany

Andreas Geier, Division of Hepatology, Department of Medicine II, University Hospital Wuerzburg, Wuerzburg 97080, Germany

Uta Merle, Department of Gastroenterology and Hepatology, University Hospital Heidelberg, Heidelberg 69120, Germany

Corresponding author: Christoph G Dietrich, MD, PhD, Consultant Physician-Scientist, Gastro-Praxis Wiesbaden, Leberzentrum Wiesbaden, Langenbeckplatz 2, Wiesbaden 65189, Germany. christoph.g.dietrich@gmail.com

Abstract

The pandemics of coronavirus disease 2019 (COVID-19) and non-alcoholic fatty liver disease (NAFLD) coexist. Elevated liver function tests are frequent in COVID-19 and may influence liver damage in NAFLD, while preexisting liver damage from NAFLD may influence the course of COVID-19. However, the prognostic relevance of this interaction, though, is unclear. Obesity is a risk factor for the presence of NAFLD as well as a severe course of COVID-19. Cohort studies reveal conflicting results regarding the influence of NAFLD presence on COVID-19 illness severity. Striking molecular similarities of cytokine pathways in both diseases, including postacute sequelae of COVID-19, suggest common pathways for chronic low-activity inflammation. This review will summarize existing data regarding the interaction of both diseases and discuss possible mechanisms of the influence of one disease on the other.

Key Words: COVID-19; Postacute sequelae of COVID-19; Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Inflammation; Fatty liver

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Core Tip: The "colliding" pandemics of coronavirus disease 2019 (COVID-19) and non-alcoholic fatty liver disease (NAFLD) influence each other in several ways. Molecular similarities of cytokine pathways in both diseases including postacute sequelae of COVID-19 (PASC) may be responsible for amplification of chronic low-active inflammation. While there are conflicting data regarding the clinical influence of NAFLD on acute COVID-19 and vice versa, further research is necessary to study the long-term influence of COVID-19 hygienic measures and PASC on NAFLD.

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INTRODUCTION

The prevalence of non-alcoholic fatty liver disease (NAFLD) has increased rapidly over the past 30 years, particularly in Western countries. This is due to a lifestyle with hypercaloric diets and obesity leading to a concomitant increase in metabolic syndrome[1]. It is estimated that approximately 30% of people in Western countries have NAFLD, and approximately 5% have non-alcoholic steatohepatitis (NASH), the inflammatory variant of fatty liver[2]. NAFLD and NASH represent chronic liver diseases with high morbidity and potential mortality.

The coronavirus disease 2019 (COVID-19) pandemic began in Wuhan, China, in late 2019. From there, the disease spread rapidly throughout the world. To date, over 500 million people have contracted COVID-19 and over 6 million people have died from it[3]. Despite effective vaccination, it is foreseeable that the coronavirus cannot be eradicated. While vaccination protects against a severe course, it cannot completely prevent infection and minor disease. To that extent, COVID-19 is likely to persist in the world as a disease, its severity depending on the prevailing variants, and to impact the population and preexisting concomitant diseases in an individual. After acute COVID-19 resolves, a proportion of COVID-19 patients suffer from postacute sequelae of COVID-19 (PASC) also named "long COVID" - as a range of new, returning, or ongoing health problems people can experience four or more weeks following initial severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection[4,5]. Therefore, in addition to acute COVID-19, postacute sequelae from COVID-19 are an emerging global health crisis, and there are hints that metabolic factors and a chronic inflammatory state (both characteristics of NAFLD) predispose patients to PASC[6].

While NAFLD is a noninfectious disease whose pandemic spread depends on people's lifestyle, especially dietary habits, COVID-19 has an acute course due to its nature as an infectious disease. This leads to waves of infection that have prompted epidemic hygiene countermeasures to contain the infection, especially in the past 2 years (2020 and 2021). Lockdowns have occurred in numerous countries to restrict the mobility of people and thus prevent the spread of the coronavirus. The harsh isolation and lockdown measures in the past 2 years under the more pathogenic previous variants had significant sociological and psychological effects. Thus, in addition to the direct viral effects of COVID-19 on the liver, there are also indirect effects on the liver or liver disease, which may play an important role in the further development of these diseases. As COVID-19 can still cause renewed lockdowns and isolation measures in the future, for example, when more lethal mutations arise again or due to newly emerging infectious diseases, such effects should also be modeled and taken into account in the future.

NAFLD and COVID-19 can be referred to as "syndemic"[7]. They represent, in a sense, "colliding pandemics"[8] due to the various possible interactions, which have different dynamics but some molecular and pathogenetic commonalities. These effects are summarized in this review and the current state of the evidence is evaluated. Between August and September 2022, we searched PubMed using the terms coronavirus, COVID-19, SARS-CoV-2, NAFLD, fatty liver, NASH, MAFLD. We analyzed all retrieved abstracts and obtained the full papers, if the study was dedicated to the connection between COVID-19 and NAFLD.

MOLECULAR SIMILARITIES OF BOTH DISEASES

NAFLD covers a wide spectrum of severity, ranging from bland fatty liver without any inflammation (NAFL) and with little or no tendency to progress all the way to NASH with inflammatory reactions and hepatocyte damage with or without fibrosis. A total of 5% to 20% of patients with NAFLD develop NASH, which undergoes a further transition to higher-grade fibrosis and eventually liver cirrhosis in 10% to 20% of cases[9]. These clinical features of NAFLD are the background for chronic low inflammatory activity of the disease. Intestinal barrier dysfunction plays a major role in triggering and

amplifying these inflammatory processes, leading to translocation of bacteria or bacterial components into the portal circulation and induction of hepatic inflammation[10]. Obesity induced by an unhealthy lifestyle (insufficient exercise and hypercaloric diet) leads to increased secretion of proinflammatory leptin, interleukin (IL)-6, and tumor necrosis factor (TNF)-α from peripheral adipose tissue, while secretion of adiponectin, an inhibitor of human stellate cell proliferation, is decreased[11]. The massive disruption of lipid metabolism due to the disturbed balance between lipolysis, oxidation, secretion, and uptake of lipids between adipose tissue and liver contributes to hepatic steatosis as well as lipotoxicity, affecting key cellular elements such as the endoplasmic reticulum or mitochondrial function[12]. In terms of a vicious cycle, hepatic metabolic pathways (especially β-oxidation) are dysregulated and further reinforce the imbalance in lipid metabolism[13] and thus lipotoxicity. Activation of human stellate cells and cytokine production by Kupffer cells follows, with IL-1β, TNF-α, IL-6, interferon (IFN)-γ, nuclear factor-kappaB, and reactive oxygen species being key extracellular and/or intracellular proinflammatory mediators that maintain chronic low-activity inflammation and induce the development of fibrosis[14,15].

Interestingly, several of these factors also appear to play important roles in COVID-19 pathogenesis in the context of systemic inflammatory response syndrome. IL-1β, TNF-α, IL-6, and IFN-γ are elevated during acute COVID-19 disease[16], and IL-6 in particular may be considered a central cytokine for the hepatic effects of COVID-19 due to its principal role in the negative acute phase response. After acute COVID-19 resolves, chronic systemic inflammatory responses may persist in patients with sequelae after acute disease, although the exact molecular drivers of PASC are largely unknown. Recently, Schultheiß *et al*[17] showed that PASC is associated with chronic elevation of IL-1β, TNF-α, and IL-6 levels. Phetsouphanh *et al*[18] even demonstrated elevation of IFN-γ (and other proinflammatory cytokines) in patients 4 mo after SARS-CoV-2 infection, irrespective of whether they had PASC symptoms.

It is therefore straightforward to speculate that low-activity NAFLD inflammation may be amplified or exacerbated by the acute phase of COVID-19 and chronic systemic inflammatory responses in at least some patients after acute COVID-19, resulting in interactions between the two diseases at the molecular level (see Figure 1).

CLINICAL EFFECTS OF COVID-19 ON THE LIVER AND THEIR MECHANISMS

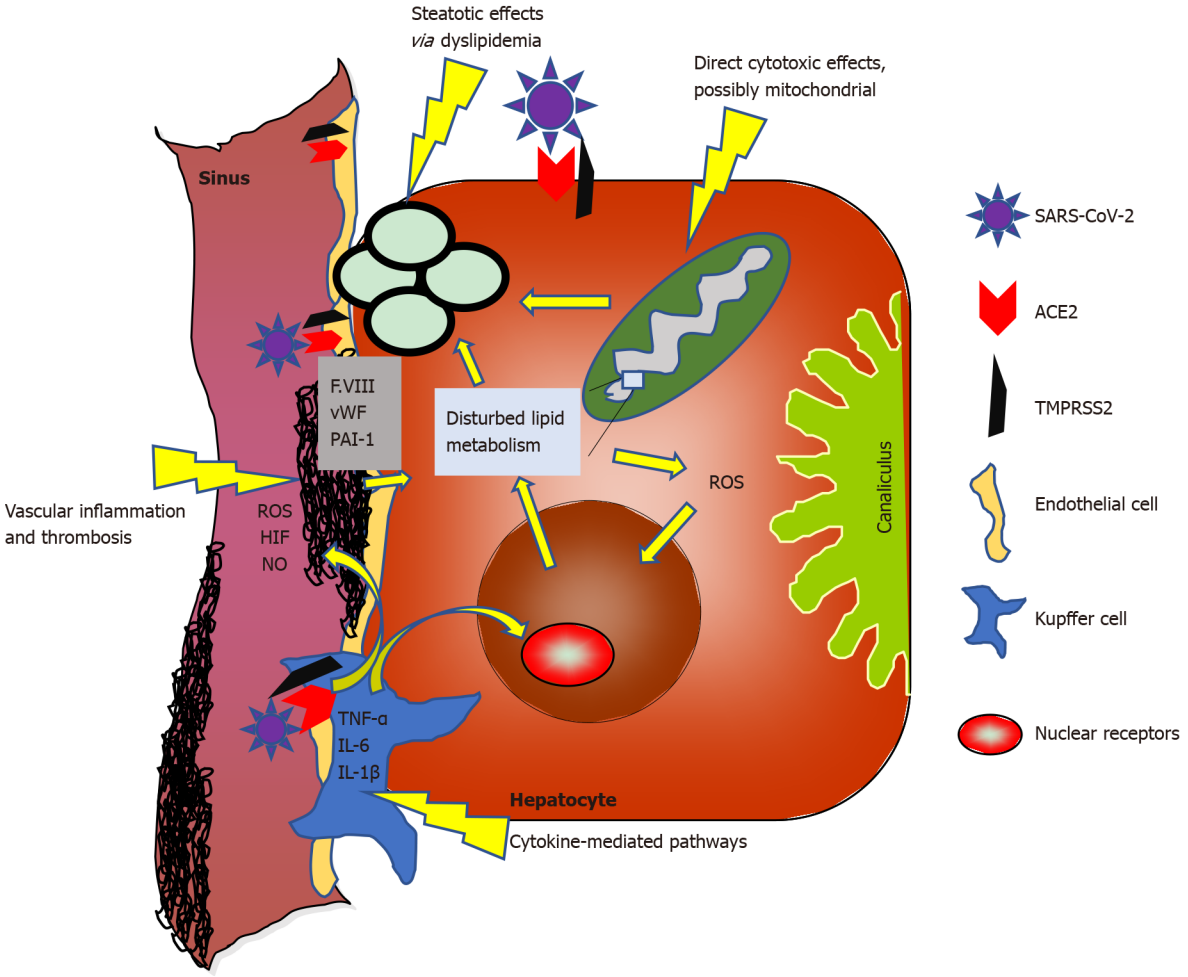
Because COVID-19 is an aerosol-transmitted disease, clinical symptoms of respiratory disease have always been the primary clinical focus. However, many case series and clinical studies show that COVID-19 also has systemic effects. These include vascular inflammation, thrombosis, and other organ involvement. Liver inflammation is therefore only part of a systemic inflammatory component of SARS-CoV-2 and is almost never the primary clinical symptom.

In a comprehensive review, it was shown that COVID-19 leads to elevations in liver enzymes in approximately 17%-58% of patients[19]. Elevations of transaminases ("hepatitis") dominate, and cholestatic constellations are much less frequent, suggesting predominantly hepatocytic damage[19]. Frequently, this concomitant COVID hepatitis is clinically inapparent. In a recent meta-analysis involving over 77000 patients, the prevalence of clinically overt liver damage was shown to be correlated with the severity of COVID-19. In this analysis, liver damage was described in 40%-47% of severe COVID-19 cases, whereas patients with a milder course were affected in only 10% on average [20]. COVID-19 may trigger acute-on-chronic liver failure in patients with liver cirrhosis due to NAFLD [21]. In contrast, severe hepatic inflammation with impairment of liver function does not seem to occur in patients without advanced preexisting fibrosis[22].

Intracellular uptake of SARS-CoV-2 requires binding of the virus with the spike protein to angiotensin-converting enzyme 2 (ACE2). Further molecular interactions with transmembrane serine protease 2, among others, lead to priming of the S protein and internalization of the virus and its genetic material into the cell[23].

Hepatic tropism of SARS-CoV-2 has been shown recently[24]. However, the exact mechanism of infection of the liver is unclear. Although ACE2 protein expression was observed in the liver, this expression is predominantly located in Kupffer cells (and only at relatively low levels in hepatocytes). In line with that, the SARS-CoV-2 spike protein could be detected in Kupffer and parenchymal cells (for example, hepatocytes)[24,25]. As a potential mechanism of hepatocyte infection, alternative hepatocyte cell entry facilitators are under discussion, *e.g.*, high-density lipoprotein scavenger receptor class B member 1[24] and the asialoglycoprotein receptor[26].

Several mechanisms of liver cell damage are conceivable (see also Figure 1). A direct cytotoxic effect does not appear to be the dominant mechanism of damage in the normal liver. Healthy hepatocytes express almost no ACE2, whereas in liver cirrhosis, ACE2 expression and activity are significantly higher[27]. These molecular regulatory mechanisms tend to argue against direct cytotoxic effects of SARS-CoV-2 in healthy liver but could explain why cirrhotic patients are more susceptible to (further) liver damage or more severe COVID-19 disease overall.



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Figure 1 Possible mechanisms of coronavirus disease-induced liver injury and the interplay between molecular pathways of inflammation in both diseases (in pre-existing non-alcoholic fatty liver disease). F. VIII: Factor VIII; vWF: von Willebrand factor; HIF: Hypoxemia-inducible factor; IL-1β: Interleukin-1β; IL-6: Interleukin-6; NO: Nitric oxide; PAI-1: Plasminogen activator inhibitor 1; ROS: Reactive oxygen species; TNF: Tumor necrosis factor; SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; ACE2: Angiotensin converting enzyme-2; TMPRSS2: Transmembrane serine protease 2.

Inflamed hepatocytes (as well as other somatic cells) may exhibit mitochondrial dysfunction in NAFLD or NASH[28,29], which in turn favors ACE2 upregulation[30] and could support viral infection. Conversely, COVID-19 also appears to directly affect mitochondrial function[31]; thus, these effects may amplify each other and induce a more severe course of both diseases.

Another pathogenetic mechanism is the additional fat storage in hepatocytes triggered by SARS-CoV-2. COVID-19 causes dyslipidemia[32] and autopsy studies show a high proportion of steatosis in COVID-19 patients[33-35]. However, it remains unclear in many cases whether steatosis was already preexisting or triggered upon infection. NAFLD patients apparently express ACE2 and various serine proteases at higher levels in the liver[36]; thus, preexisting steatosis may promote COVID-19-induced damage. In turn, COVID-19 may exacerbate steatosis. Whether these dynamics are quantitatively significant effects must remain open for now and deserves future research.

The most significant hypothesis of hepatocyte injury based on clinical characteristics concerns inflammatory cytokine-mediated pathways[37], with commonalities between acutely mediated COVID-19 effects and NAFLD-mediated chronic liver inflammation (especially IL-6; see above). ACE2 expression on Kupffer cells[38] may be the origin of virus-mediated, locally amplified liver inflammation.

Autopsy studies also support possible vascular-associated mechanisms for COVID-19-mediated liver injury[35,39]. One early series of postmortem liver biopsies from patients with COVID-19 reported portal or sinusoidal vascular thrombosis in at least 50% of patients[39]. Patients with COVID-19 exhibit coagulopathy and endotheliopathy, characterized by elevated levels of von Willebrand factor and factor VIII[40]. As factor VIII is produced primarily by liver sinusoidal endothelial cells (LSECs)[41], hypercoagulable LSECs might play an additional role in COVID-19-related liver injury. Of note, endotheliopathy has been reported to be sustained following COVID-19[42], suggesting not only acute but also long-term interactions and consequences of endothelial-mediated inflammation with chronic liver

diseases such as NAFLD. Another direct link between COVID-19 and NAFLD may be *via* plasminogen activator inhibitor 1 (PAI-1). PAI-1 has been shown to be elevated among COVID-19 patients[43]. This role of PAI-1 in COVID-19 liver injury is potentially interesting, especially in NAFLD patients, as elevated PAI-1 has been associated with NAFLD and NASH[44]; therefore, COVID-19-induced PAI-1 elevation may aggravate NAFLD.

The mechanisms described above partly overlap or influence each other, as indicated by the arrows in Figure 1. The pathogenic significance of these molecular associations is not clear in most cases. There are many hints to suggest that the effects of COVID-19 on the liver and especially NAFLD are multifactorial and may also differ individually. Despite these different possible mechanisms, severe liver injury and liver failure are rare even in prediseased liver patients[22].

INFLUENCE OF NAFLD ON COVID-19

Numerous studies of varying quality have addressed the risk for morbidity and mortality of COVID-19 in subjects with NAFLD (Table 1). The studies are extraordinarily heterogeneous, making reliable conclusions difficult. Numerous studies were published only as letters to the editor, not as full articles. Almost all studies are retrospective and include low numbers of patients. Definitions of NAFLD/metabolic syndrome-associated fatty liver disease (MAFLD) vary considerably; in some cases, only blood-based surrogate scores for steatosis and liver fibrosis (such as the hepatic steatosis index, NAFLD fibrosis score, or Dallas steatosis index) were applied. Imaging with ultrasound or computed tomography (CT) is mostly used as the criterion for the presence of fatty liver. Biopsy-proven NAFLD is a rare exception. Importantly, data on alcohol consumption are lacking in many studies. No statements are found on inflammatory activity of fatty liver (*i.e.*, on the presence of NASH). Definitions of severe COVID progression are also inconsistent. Studies that define NAFLD only by scores or by imaging (ultrasound, CT) during the course of hospitalization for COVID-19 cannot provide information about the presence of fatty liver before COVID-19 emergence. Control groups almost invariably contain fewer patients with classic metabolic factors, such as diabetes mellitus and obesity, than the respective NAFLD groups; this metabolic imbalance of study groups cannot easily be controlled by multivariate analysis.

These conditions do not allow confident conclusions to be drawn at this time. Presently, the data suggest that NAFLD alone is not a relevant risk factor for severe COVID-19 progression or mortality. In particular, registry studies from large liver collectives with different etiologies tend not to support a special role for NAFLD[54,63,64,66]. However, available studies also show that the presence of liver fibrosis or cirrhosis is associated with a higher risk of severe COVID-19 disease[61,69]. In this respect, the increase in risk for a more severe course of COVID-19 may not be attributed to NAFLD per se but rather to advanced liver disease irrespective of the underlying etiology in general.

INFLUENCE OF EPIDEMIC HYGIENIC COVID-19 MEASURES ON NAFLD

Disease hygiene measures in the context of the infectious waves probably represent an important factor in the influence of the COVID-19 pandemic on NAFLD. The psychosocial impact of the COVID-19 pandemic resulted in measurable exacerbations of metabolic comorbidities of NAFLD. A United States cohort study examined 111 NAFLD patients and found decreases in physical activity in 51%, weight increase in 34%, and increases in alcohol consumption in 5% during the COVID-19 pandemic[72]. A Spanish study screened over 6000 workers for metabolic factors and found significant increases in body mass index, insulin resistance, and low-density lipoprotein during the pandemic. The average fatty liver index (FLI) as a surrogate for NAFLD increased from 25.2 to 33 in this study[73]. Another Spanish study showed a decrease in physical activity during lockdown with a consecutive increase in FLI and worsening of metabolic status[74]. In an Italian cohort study, 48% of 357 NAFLD patients gained weight during lockdown, and this weight gain was associated with abandonment of a Mediterranean diet and decreased physical activity in univariate analysis and various multivariate models. Interestingly, in PNPLA3-GG polymorphism patients, this genotype represented the only favoring factor for weight gain [75]. A Japanese study examined 973 patients with health checks in 2018 and 2020. In this study, the absolute number of MAFLD patients increased from 261 to 305; however, as the authors identified predominantly higher alcohol consumption as a risk factor for this development, there is actually a definition problem of MAFLD in the strict sense[76]. Overall, these studies show a decrease in physical activity and an increase in weight in the general population. It can be assumed, though not yet clearly shown, that this favors the *de novo* development or exacerbation of steatosis and inflammation in NAFLD, that fibrosis in turn may be further advanced and that the prognosis of the liver disease overall is thus worsened at the end. Long-term studies into these effects of pandemic-associated lifestyle changes are necessary.

Table 1 Studies with data regarding the risk of non-alcoholic fatty liver disease/metabolic syndrome-associated fatty liver disease for severe coronavirus disease 2019

Ref.	Study type and number of NAFLD patients	Results	Appraisal
Zhou *et al*[45], 2020	Retrospective, matched cohorts, *n* = 55 per group	More severe COVID-19 in MAFLD OR = 4.07	Poor matching regarding metabolic status, more male pat in MAFLD group
Targher *et al*[46], 2020	Retrospective, cohort study *n* = 94 (216 w/o MAFLD)	More severe COVID-19 with higher FIB-4 or NFS	No matching, no full paper
Ji *et al*[47], 2020	Retrospective, cohort study *n* = 202	NAFLD 87 % in progressive COVID-19 (*n* = 39) *vs* 26 % in stable COVID-19 (*n* = 163)	Comorbidities highly different between groups, no full paper, NAFLD definition only *via* HSI
Hashemi *et al*[48], 2020	Retrospective, CLD cohort with 55 NAFLD patients (294 w/o CLD/NAFLD)	Presence of CLD and NAFLD higher risk for mechanical ventilation (OR = 2.15) and ICU admission (OR = 2.3), cirrhosis risk factor for mortality	Imbalance in metabolic status, NAFLD diagnosis relying on prior imaging
Huang *et al*[49], 2020	Retrospective, cohort *n* = 86 (194 w/o NAFLD)	Only higher ALT in NAFLD patients, course of disease comparable to controls	NAFLD only defined by HSI, imbalance in metabolic status
Forlano *et al*[50], 2020	Retrospective, cohort *n* = 61 (132 w/o NAFLD)	NAFLD pat with higher CRP, younger age. Fibrosis or cirrhosis no risk for more severe COVID-19	Only hospitalized patients, higher BMI in NAFLD, diagnosis by imaging (US or CT)
Lopez-Mendez *et al* [51], 2021	Retrospective, cohort study *n* = 66 (89 w/o steatosis)	Presence of steatosis (and/or liver fibrosis) not related to severity or mortality of COVID-19	Steatosis only defined by HSI, imbalance on metabolic status
Zheng *et al*[52], 2020	Retrospective, cohort study *n* = 66 (45 with and 21 w/o obesity)	Obesity risk factor for COVID severity in MAFLD patients (OR = 6.3)	Diagnosis of MAFLD by CT and clinical criteria, no controls w/o MAFLD, no full paper
Zhou *et al*[53], 2020	Retrospective, cohort study *n* = 93 (out of 327 total patients)	Younger MAFLD patients with relatively higher risk for severe COVID	No full paper, small number of older patients, CT data
Valenti *et al*[54], 2020	Retrospective, United Kingdom Biobank cohort (Mendelian randomization), total *n* > 500000	No evidence for NAFLD as risk factor for severe COVID-19	Data errors possible, partly little characterization of patients, no full paper
Mahamid *et al*[55], 2021	Retrospective, cohort study *n* = 22 (49 w/o MAFLD)	8/22 with severe COVID-19 *vs* 5/49 w/o MAFLD	CT data, large differences in metabolic status between groups
Chen *et al*[56], 2021	Retrospective, cohort study *n* = 178 (164 w/o hepatic steatosis)	More intubation and vasopressors in steatosis, but lower mortality	Only hospitalized patients, HSI or imaging, rel. high percentage of steatosis in cohort, metabolic status not balanced
Gao *et al*[57], 2021	Retrospective, matched cohorts, *n* = 65	OR = 4.07 for severe COVID-19 only in non-diabetic patients	Poor matching regarding metabolic status, NAFLD diagnosis by CT and clinical criteria, duplicate patients with Zhou *et al*[45]
Marjot *et al*[58], 2021	Retrospective CLD cohort with 322 NAFLD patients	No higher mortality for NAFLD patients in multivariate analysis	Control group matched only to complete CLD cohort, not specifically to NAFLD patients. Unclear definition of NAFLD
Parlak *et al*[59], 2021	Retrospective, cohort study *n* = 55 (288 w/o fatty liver)	Presence of fatty liver risk factor (OR = 3.9) for severe COVID-19	CT data, no data regarding BMI, no data comparison NAFLD *vs* non-NAFLD
Mushtaq *et al*[60], 2021	Retrospective, cohort study *n* = 320 (269 w/o NAFLD)	NAFLD predictor for mild or moderate liver injury, but not for disease severity or mortality	NAFLD only defined by HIS, imbalance on metabolic status, no full paper
Campos-Murguía *et al*[61], 2021	Retrospective, cohort study *n* = 176 (256 w/o MAFLD)	Liver fibrosis, not MAFLD alone, predictor for severity and mortality of COVID-19	CT data, relatively good obesity matching to controls
Kim *et al*[62], 2021	Retrospective, CLD cohort with 456 NAFLD patients	NAFLD no risk factor for severe course or mortality of COVID-19	No control cohort w/o liver disease, tertiary centers only, NAFLD ICD-diagnosis
Simon *et al*[63], 2021	Large Swedish CLD cohort (total *n* = 42320), biopsy confirmed, with unclear number of NAFLD patients	CLD presence as risk factor for hospitalization, but not for severe COVID (including cirrhosis)	Historic cohort with possible drop-outs, underlying CLD in controls may have been missed
Roca-Fernández *et al* [64], 2021	United Kingdom Biobank cohort, with prospective data on infection and hospitalization for COVID	Fatty liver with increased risk for testing COVID-positive, obesity and fatty liver with higher risk for hospitalization, but not obesity alone	Data errors possible, little characterization of patients, small number of patients with severe COVID

Ziaee *et al*[65], 2021	Retrospective Iranian cohort *n* = 218 (357 patients w/o NAFLD, additional control group w/o COVID)	Fatty liver significant more prevalent in COVID group compared to control group (38% *vs* 9%). Longer hospital stay and larger pulmonal involvement in NAFLD patients	Very low percentage of fatty liver in control group. Control group with missing data
Liu *et al*[66], 2022	COVID-19 HGI and United Kingdom Biobank cohorts (Mendelian randomization), retrospective data, total *n* > 2500000	No evidence for NAFLD as risk factor for severe COVID-19	Data errors possible, little characterization of patients, no full paper
Chang *et al*[67], 2022	South Korean COVID-19 cohort with FLI score (total *n* = 3122)	Highest FLI tertile with higher risk for severe COVID-19, but not for higher mortality	No NAFLD-specific case definition, FLI score tertile cutoff low
Vrsaljko *et al*[68], 2022	Prospective cohort study *n* = 120 (96 w/o NAFLD)	NAFLD with higher risk for severe COVID-19 including pulmonary thrombosis	No data regarding fibrosis
Tripon *et al*[69], 2022	Retrospective French cohort *n* = 311 (408 w/o NAFLD)	NAFLD with higher risk for hospitalization, high FIB-4 with higher risk for severe COVID-19	NAFLD only defined by NFS, important data missing in cohort patients
Moctezuma-Velázquez *et al*[70], 2022	Retrospective Mexican cohort *n* = 359 (111 w/o NAFLD)	NAFLD associated with mortality, ICU admission and mechanical ventilation, but CT-determined liver steatosis was not	NAFLD definition based on DSI, small number of control patients, only hospitalized patients
Okuhama *et al*[71], 2022	Retrospective Japanese cohort *n* = 89 (133 w/o fatty liver)	Fatty liver associated with severe COVID-19	CT data, no data regarding dyslipidemia, only hospitalized patients

BMI: Body mass index; CLD: Clinical liver disease; CT: Computed tomography; DSI: Dallas steatosis index; FLI: Fatty liver index; HIS: Hepatic steatosis index; MAFLD: Metabolic syndrome-associated fatty liver disease; NFS: Non-alcoholic fatty liver disease fibrosis score; pat: Patients; US: Ultrasound; NAFLD: Non-alcoholic fatty liver disease; OR: Odds ratio; ICU: Intensive care unit; COVID-19: Coronavirus disease 2019; HGI: Haemoglobin glycation index; ICD: International Classification of Diseases; CRP: C-reactive protein; ALT: Alanine aminotransferase.

CONCLUSION

NAFLD and COVID-19 have both taken a pandemic course in their own ways. Whereas infectious disease essentially causes short-term disease, NAFLD represents a chronic pandemic. Interestingly, the molecular mechanisms of inflammation are similar, although NAFLD is more of a chronic low-activity inflammation while COVID-19 is an acute inflammatory condition. However, in NAFLD patients with ongoing PASC both conditions may chronically interact with unknown mutual effects.

Obesity certainly represents an important unifying clinical factor of both diseases, as obesity is an important risk factor for the development of NAFLD and the severe course of COVID-19. In contrast, the presence of NAFLD per se does not appear to be a relevant risk factor for particularly severe COVID-19. The effects of COVID-19 on liver disease are more complex and still poorly understood. While the direct viral effect on NAFLD may be limited, probably because of the short duration of the acute viral infection, the individual effects associated with lockdowns and isolation are potential risk factors for disease progression due to a reported decrease in physical activity together with an increase in obesity. European Association for the study of the liver position papers provide valuable recommendations for liver patients after the outbreak of the pandemic, including specific recommendations for NAFLD patients[77,78].

FOOTNOTES

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Country/Territory of origin: Germany

ORCID number: Christoph G Dietrich 0000-0001-6927-7970; Andreas Geier 0000-0002-9626-5083; Uta Merle 0000-0003-1386-3350.

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Aviv Pudipeddi, Caroline Fung, Britt Christensen, Robert V Bryant, Kavitha Subramaniam, John Chetwood, Sudarshan Paramsothy, Rupert W Leong

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Aviv Pudipeddi, John Chetwood, Sudarshan Paramsothy, Rupert W Leong, Gastroenterology and Liver Services, Concord Repatriation General Hospital, Sydney 2139, Australia

Aviv Pudipeddi, Sudarshan Paramsothy, Rupert W Leong, Faculty of Medicine and Health, Concord Clinical School, University of Sydney, Sydney 2138, Australia

Caroline Fung, Department of Anatomical Pathology, Concord Repatriation General Hospital, Sydney 2139, Australia

Britt Christensen, Department of Gastroenterology, Royal Melbourne Hospital, Melbourne 3050, Australia

Britt Christensen, Department of Medicine, University of Melbourne, Melbourne 3052, Australia

Robert V Bryant, Department of Gastroenterology and Hepatology, The Queen Elizabeth Hospital, Adelaide 5011, Australia

Kavitha Subramaniam, Gastroenterology and Hepatology Unit, Canberra Hospital, Canberra 2605, Australia

Kavitha Subramaniam, Australian National University Medical School, Australian National University, Canberra 2601, Australia

Sudarshan Paramsothy, Rupert W Leong, Faculty of Medicine and Health Sciences, Macquarie University Hospital, Sydney 2109, Australia

Corresponding author: Aviv Pudipeddi, FRACP, MBBS, Doctor, Gastroenterology and Liver Services, Concord Repatriation General Hospital, Level 1West ACE Unit, Concord Repatriation General Hospital, Hospital Road, Concord, Sydney 2139, Australia.
avivpudipeddi@gmail.com

Abstract

BACKGROUND

Histological remission is increasingly accepted as a treatment endpoint in the

management of ulcerative colitis (UC). However, the knowledge of histology guidelines and the attitudes towards their use in clinical practice by gastroenterologists and pathologists is unknown.

AIM

To evaluate the knowledge of histology guidelines and attitudes towards the use of histology in UC by gastroenterologists and pathologists.

METHODS

A prospective, cross-sectional nationwide survey of gastroenterologists and pathologists who analyse UC specimens was conducted. The survey consisted of 34 questions to assess gastroenterologists' and pathologists' knowledge (score out of 19) and attitudes towards histological assessment in UC. Survey questions were formulated using the European Crohn's and Colitis position paper on histopathology and the British Society of Gastroenterology biopsy reporting guidelines. It included knowledge of histological assessment of disease activity and dysplasia, knowledge of histological scoring systems for ulcerative colitis, uptake of histology scoring systems in routine practice, attitudes towards the role of histological activity, and the use of histological activity in clinical scenarios.

RESULTS

Of 89 responders (77 gastroenterologists, 12 pathologists), there was almost universal acceptance that histological assessment should form part of UC evaluation [95% gastroenterologists, 92% pathologists]. However, gastroenterologists reported that 92% of their pathologists do not use a histological scoring system. Utilisation of a formal histological scoring system was preferred by 77% of gastroenterologists and 58% of pathologists. Both groups lacked awareness of the Geboes Score, Nancy Index and Robarts Histopathological Index scoring systems with 91%, 87%, and 92% of gastroenterologists respectively; and 83%, 83%, and 92% pathologists respectively, being uncertain of scoring systems' remission definitions. Histology knowledge score was not significantly different between gastroenterologists and pathologists [9/19 (IQR: 8-11) *vs* 8/19 (IQR: 7-10), $P = 0.54$]. Higher knowledge scores were predicted by hospital attending gastroenterologists ($P = 0.004$), participation in inflammatory bowel disease (IBD) multidisciplinary teams ($P = 0.009$), and self-declared IBD sub-specialist ($P = 0.03$).

CONCLUSION

Histological remission is a recognised target for both gastroenterologists and pathologists. Despite this, knowledge of histological scoring systems and their utilisation is poor.

Key Words: Histology; Scoring system; Ulcerative colitis; Survey

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Core Tip: This manuscript describes, for the first time, the knowledge and attitudes of gastroenterologists and pathologists towards the use of histology in clinical practice. Given the increasing literature and use of histology in trials, there is a need to understand the current perceptions of using histology in the real-world. Using a novel Inflammatory Bowel Disease Knowledge score, we demonstrate that although histology is an accepted endpoint, knowledge is poor, particularly relating to histological scoring systems. As such, these results illustrate a pressing need and opportunity to improve knowledge around histology scores amongst gastroenterologists and pathologists and develop consensus agreements on a reporting approach.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory disease characterised by a relapsing and remitting course[1]. Disease activity is typically evaluated using clinical, biochemical and endoscopic assessments.

Treatment goals have evolved over time, and current consensus guidelines from the Selecting Therapeutic Targets in Inflammatory Bowel Disease initiative (STRIDE-II) recommend achieving clinical and endoscopic remission[2]. However, up to 40% of patients who achieve these therapeutic endpoints may have persistent histological inflammatory activity[3,4].

Despite endoscopic normalization, ongoing active histological activity may be associated with poorer clinical outcomes including higher clinical relapse rates, corticosteroid requirement, hospitalization, colectomy and development of colorectal neoplasia[3-7]. Although histological remission is currently not a formal treatment target by consensus expert-opinion, STRIDE-II guidelines do recommend that formal histological assessment take place to determine the depth of remission and help prognosticate patient outcomes. Further, it is increasingly incorporated into clinical drug trials, with central reading to reduce bias, to provide objective scoring of inflammatory activity[2]. Standardized histological scoring systems with varying levels of validity have been developed to quantify the degree of microscopic inflammatory activity and provide a more accurate assessment of mucosal inflammation[8-12]. The three most commonly used are the Geboes score, Nancy index and Robarts histopathology index due to evidence of their content validity and reliability in evaluating histological features[13].

Although accepted in modern clinical drug trials and research settings, histological disease activity and scoring systems have not been incorporated in routine clinical practice. It is not known whether gastroenterologists understand these scoring systems or if they welcome their incorporation into routine clinical care. Achieving consensus in a formal reporting scoring system will require agreement by pathologists, but their knowledge of these scoring systems and willingness to use them is also unknown. Many pathologists use written descriptions of UC activity in their reports. Whether this translates to a numerical value, if they favour a particular scoring system, or their attitude towards synaptic reporting of histological activity, is not known. This cross-sectional survey study evaluated gastroenterologist and pathologist knowledge of histological findings and scoring systems, together with their attitudes towards the role of histology in UC management. We hypothesised that based on their dedicated training, knowledge of histological scoring systems would be significantly higher in pathologists than gastroenterologists.

MATERIALS AND METHODS

Study cohort

This was a prospective cross-sectional survey of Australian gastroenterologists and pathologists from July 2021 to January 2022. Gastroenterologists were contacted by proxy through the Gastroenterological Society of Australia, and pathologists who review UC specimens were contacted by their associated gastroenterologists to participate in the survey.

Survey questionnaire and inflammatory bowel disease histology knowledge score

A survey was developed to explore the knowledge and attitudes towards the use of histology in inflammatory bowel disease (IBD) for both gastroenterologists and pathologists. The European Crohn's and Colitis Organisation (ECCO) position paper on histopathology and the British Society of Gastroenterology (BSG) biopsy reporting guidelines were utilised to formulate questions and quantify knowledge[14,15]. The structured survey was designed by a focus group of three gastroenterologists and comprised of 34 questions. It included knowledge of histological assessment of disease activity and dysplasia, knowledge of histological scoring systems for ulcerative colitis, uptake of histology scoring systems in routine practice, attitudes towards the role of histological activity, and the use of histological activity in clinical scenarios (Supplementary Data 1). Questionnaire language and ambiguity were evaluated by the focus group. A novel IBD Histology Knowledge Score was created that was derived from the survey as a tool to measure overall performance and tested for construct validity and discriminant ability (Supplementary Table 1). The IBD Histology Knowledge Score was calculated as the sum of correct responses to survey questions that aligned with the ECCO position paper on histopathology and the BSG reporting guidelines on IBD biopsies[14,15]. The maximum possible score was nineteen. For construct validity, a high-performance score had to represent a good understanding of histological findings. During the development phase, the survey was administered to senior gastroenterologists and pathologists not directly involved in designing the study, and they were deemed as criterion standards. The survey was then administered to gastroenterology fellows, junior resident medical officers and non-medical staff. Senior staff scored significantly higher (P = 0.001) than junior doctors, establishing content validity. Discriminant validity compared the knowledge scores of those who followed published guidelines *vs* those who did not.

Statistical analysis

The IBD Histology Knowledge Score was analysed as a non-parametric continuous variable, described as medians with interquartile ranges and compared using Mann-Whitney *U*-test and Kruskal-Wallis test. Parametric continuous variables were described as means and compared using the *t*-test and ANOVA test. Predictors of the IBD histology knowledge score were determined using linear regression

with backward elimination regression modelling. A *P*-value of < 0.05 was deemed statistically significant. Statistical analyses were performed with SPSS version 27 (SPSS Inc, Chicago, IL, United States).

Ethics approval

The study was approved by the Sydney Local Health District Human Research Ethics Committee (HREC CH62/6/2021-055).

RESULTS

Study cohort

A total of 89 responses were obtained, comprising 77 gastroenterologists and 12 pathologists. The response rate for gastroenterologists was 25% (n = 77/310). Subspecialty breakdown of gastroenterologists is shown in Figure 1. Gastroenterologists listed their predominant work as 31% public hospital staff specialists, 30% private practice, 21% trainee gastroenterologists, 17% visiting medical officers and 1% research-based gastroenterologist. Ninety-four percent of respondents saw > 2 IBD patients each week and 30% saw > 10 patients each week. Forty-five percent of gastroenterologists were involved in a regular IBD multidisciplinary team. Full study cohort characteristics are shown in Table 1.

Of the 12 surveyed pathologists, 83% worked in tertiary teaching hospitals and 17% were solely in private practice. Half of all pathologists were involved in regular IBD multidisciplinary meetings. Full study cohort characteristics are shown in Table 1.

Attitudes towards histology and scoring systems in UC

Histological activity was considered to have an 'emerging' or 'established' role in UC by 40% and 55% of gastroenterologists respectively. Proportions for pathologists were 33% and 58% respectively. Histological remission was considered more important to achieve than endoscopic remission by 65% of gastroenterologists ('somewhat agree' and 'agree') (Table 2).

The proportion of gastroenterologists who want to use a histological scoring system at least 'sometimes' or 'always' was 59%, and 50% for pathologists. Gastroenterologists reported that 92% of their pathologists do not routinely use a histological scoring system, whilst 83% pathologists report not routinely using a scoring system. More than half of gastroenterologists (64%) and pathologists (58%) did not know which scoring systems had undergone the most validation (Table 2).

For the Geboes score, 91% of gastroenterologists and 83% of pathologists did not know the defined histological remission score of '< 2.1'[14]. For the Nancy index, 87% of gastroenterologists and 83% of pathologists did not know the defined histological remission score of '0'[14]. For the Robarts histopathology index (RHI), 92% of gastroenterologists and pathologists did not know the defined histological remission score of '≤ 3'[14] (Table 2 and Figure 2).

Impact of histological activity on treatment decisions in clinical scenarios

The impact of histological disease activity on gastroenterologists' decisions to escalate treatment or de-escalate in particular scenarios is summarized in Table 3. In the setting of clinical and endoscopic remission, but histological activity alone, 10% of gastroenterologists would escalate therapy ('often' or 'always'). When combined with an elevated faecal calprotectin, 30% of gastroenterologists would escalate treatment. A greater proportion of gastroenterologists would de-escalate treatment if two consecutive colonoscopies showed endoscopic and histological remission, compared with a single episode of endoscopic and histological remission (53% *vs* 19% respectively). A greater proportion of gastroenterologists would aim for histological remission if a patient with UC had other risk factors for colon cancer (71%).

IBD histology knowledge score

Gastroenterologists and pathologists had similar IBD histology knowledge scores [8.0 (IQR: 6.5-10.0) *vs* 9.0 (IQR: 7.8-11.0), P = 0.54] (Table 4). Within gastroenterologists, IBD sub-specialists had higher knowledge scores compared with other gastroenterologists [10.5 (IQR: 7.3-14) *vs* 9.0 (IQR: 7.8-10.0), P = 0.02] (Figure 3A). Public hospital staff specialists had higher knowledge scores than visiting medical officers [11.0 (IQR: 9.0-13.0) *vs* 8.0 (IQR: 8.0-9.0), P = 0.003] and those in private practice [11.0 (IQR: 9.0-13.0) *vs* 8.0 (IQR: 6.3-9.8), P = 0.002] (Figure 3B). Gastroenterologists with a PhD had higher knowledge scores than those whose highest level of education was a bachelor degree [11.0 (IQR: 7.0-14.0) *vs* 9.0 (IQR: 8.0-10.0), P = 0.01] (Figure 3C). Involvement in an IBD multidisciplinary team was associated with a higher knowledge score [9.5 (IQR: 8.0-11.0) *vs* 8.0 (IQR: 6.0-10.0), P = 0.002] (Figure 3D).

On univariate analysis, subspecialty type (P = 0.005), predominant practice (p=0.004), involvement in an IBD multidisciplinary team (P = 0.002) and a higher level of education (P = 0.02) were all significantly associated with higher IBD histology knowledge scores (Table 5). On multivariate analysis, subspecialty type (P = 0.03), predominant practice (P = 0.005) and involvement in an IBD multidisciplinary team (P =

Table 1 Demographics and study cohort characteristics, *n* (%)

	Gastroenterologists (*n* = 77)	Pathologists (*n* = 12)
Age (yr)		
< 30	4 (5.2)	0 (0.0)
30-40	30 (39.0)	1 (8.3)
41-50	15 (19.5)	4 (33.3)
51-60	19 (24.7)	4 (33.3)
> 60	9 (11.7)	3 (25.0)
Location		
New South Wales	46 (59.7)	8 (66.7)
Victoria	11 (14.3)	2 (16.7)
Queensland	11 (14.3)	2 (16.7)
Western Australia	8 (10.4)	0 (0.0)
Australian Capital Territory	1 (1.3)	0 (0.0)
Highest level of education		
Bachelor of medicine/bachelor of surgery	51 (66.2)	11 (91.7)
Masters	10 (13.0)	0 (0.0)
PhD	16 (20.8)	1 (8.3)
What is your predominant practice		
Staff specialist	24 (31.2)	10 (83.3)
University academic work	1 (1.3)	0 (0.0)
Visiting medical officer	13 (16.9)	0 (0.0)
Private practice	23 (29.9)	2 (16.7)
In training program	16 (20.8)	0 (0.0)
How many IBD patients do you see each week		
0-1	5 (6.5)	N/A
2-5	31 (40.3)	N/A
6-10	18 (23.4)	N/A
> 10	23 (29.9)	N/A
Involved in regular IBD multidisciplinary meeting		
Yes	35 (45.5)	6 (50.0)
No	42 (54.5)	6 (50.0)

IBD: Inflammatory bowel disease; N/A: Not applicable.

0.009) remained significant predictors for higher IBD histology knowledge scores (Table 5).

DISCUSSION

Therapeutic goals in UC have evolved from achieving clinical response to attaining objective targets of resolution of inflammation beyond symptoms such as biochemical and endoscopic remission. However, histological remission outside of the research setting has yet to be adopted by gastroenterologists and pathologists. Our study revealed firstly that histological activity is a recognised treatment goal for gastroenterologists who wish to use histology results in combination with other endpoints to guide management decisions. Secondly and conversely, despite this awareness and use of histology, there is a poor knowledge of histological scoring systems in UC not only by gastroenterologists, but by pathologists as well. As such there is an opportunity to develop consensus guidelines incorporating

Table 2 Attitudes towards histology and histological scoring systems, *n* (%)

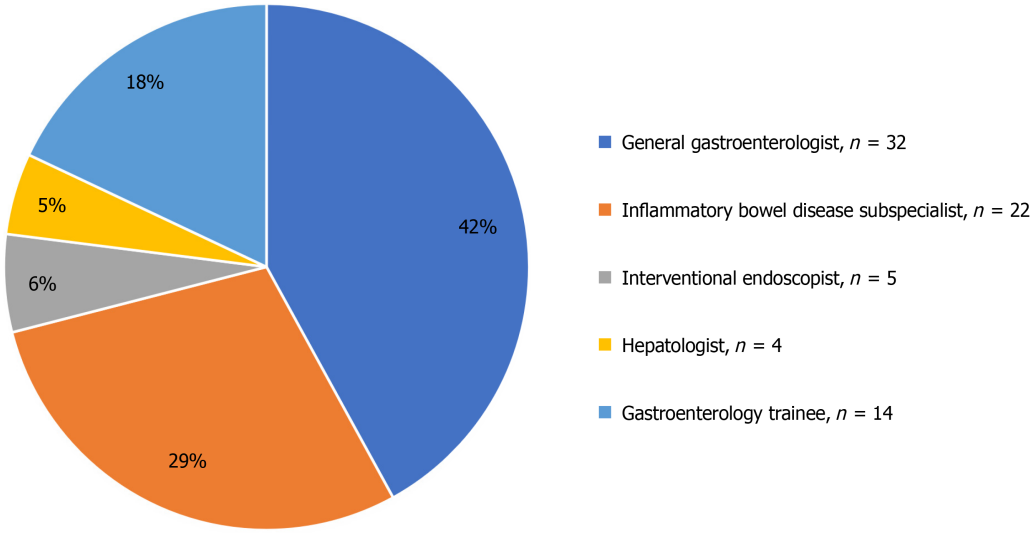
	Gastroenterologists (*n* = 77)	Pathologists (*n* = 12)
The role of histological activity in IBD is		
Not established	3 (3.9)	1 (8.3)
Preliminary	1 (1.3)	0 (0.0)
Emerging	31 (40.3)	4 (33.3)
Established	42 (54.5)	7 (58.3)
Histological remission is more important to achieve than endoscopic remission		
Disagree	4 (5.2)	N/A
Somewhat disagree	13 (16.9)	N/A
Neither agree nor disagree	10 (13.0)	N/A
Somewhat agree	36 (46.8)	N/A
Agree	14 (18.2)	N/A
What histological scoring system does your pathologist routinely or frequently use in their reports		
Geboes	2 (2.6)	0 (0.0)
Nancy index	3 (3.9)	1 (8.3)
RHI	1 (1.3)	0 (0.0)
They do not routinely use a scoring system	71 (92.2)	10 (83.3)
Other		IBD-DCA score (*n* = 1)
I would like to use a histological scoring system for my IBD patients		
Never	8 (10.4)	4 (33.3)
Rarely	10 (13.0)	1 (8.3)
Occasionally	14 (18.2)	1 (8.3)
Sometimes	23 (29.9)	3 (25.0)
Always	22 (28.6)	3 (25.0)
Which scoring systems have undergone the most validation		
Modified Riley score	1 (1.3)	1 (8.3)
Geboes score	13 (16.9)	3 (25.0)
Nancy index	20 (26.0)	5 (41.7)
RHI	9 (11.7)	3 (25.0)
Truelove and Richards score	5 (6.5)	0 (0.0)
Not sure	49 (63.6)	7 (58.3)
What Geboes score is considered histological remission		
< 1.1	2 (2.6)	1 (8.3)
< 2.1	7 (9.1)	2 (16.7)
< 3.1	4 (5.2)	0 (0.0)
< 4.1	1 (1.3)	0 (0.0)
Not sure	63 (81.8)	9 (75.0)
What Nancy index is considered histological remission		
0	10 (13.0)	2 (16.7)
≤ 1	4 (5.2)	3 (25.0)

≤ 2	0 (0.0)	0 (0.0)
≤ 3	0 (0.0)	0 (0.0)
Not sure	63 (81.8)	7 (58.3)
What Robarts histopathology index is considered histological remission		
≤ 2	4 (5.2)	1 (8.3)
≤ 3	6 (7.8)	1 (8.3)
≤ 4	0 (0.0)	0 (0.0)
≤ 5	0 (0.0)	1 (8.3)
Not sure	67 (87.0)	9 (75.0)

RHI: Robarts histopathology index; IBD: Inflammatory bowel disease; N/A: Not applicable.

Table 3 Impact of histological disease activity on treatment management in clinical scenarios, *n* (%)

Scenario	Never	Not often	Sometimes	Often	Always
If a patient is in clinical and endoscopic remission, but has histological activity, then I will escalate medical therapy	14 (18.2)	35 (45.5)	20 (26.0)	5 (6.5)	3 (3.9)
If a patient is in clinical and endoscopic remission, but has an elevated faecal calprotectin (> 100 μg/g) and histological activity, then I will escalate medical therapy	4 (5.2)	18 (23.4)	31 (40.3)	19 (24.7)	5 (6.5)
If a patient is in clinical, endoscopic and histological remission, (but prior colonoscopy showed Mayo 1 endoscopic disease), then I will de-escalate medical therapy	7 (9.1)	19 (24.7)	36 (46.8)	15 (19.5)	0 (0.0)
If a patient is in clinical remission, with their last 2 colonoscopies showing endoscopic and histological remission, then I will de-escalate medical therapy	2 (2.6)	2 (2.6)	31 (40.3)	38 (49.4)	4 (5.2)
If a patient with ulcerative colitis has other risk factors for colon cancer, then I will aim to achieve histological remission	0 (0.0)	7 (9.1)	14 (18.2)	27 (35.1)	29 (37.7)



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Figure 1 Subspeciality characteristics of gastroenterologists.

gastroenterologists and pathologists that are adopted by the respective societies to further this evolving field.

Our study showed 95% of gastroenterologists believe histological activity plays a role in the management of UC, with 76% wanting to use a histological scoring system in clinical practice. Further evidence on the role of UC histological activity scores is required as only a small proportion of gastroenterologists currently make treatment decisions based solely on histological activity. In UC patients with clinical and endoscopic remission but ongoing histological disease activity, 10% of gastroenterologists

Table 4 Inflammatory bowel disease histology knowledge scores

	Gastroenterologists (*n* = 77)	Pathologists (*n* = 12)
IBD histology knowledge score [median (IQR)]	9.0 (7.8-11.0)	8.0 (6.5-10.0)
Type of subspecialist		
General gastroenterologist	8.0 (7.0-9.0)	N/A
IBD subspecialist	10.5 (7.3-14)	N/A
Interventional endoscopist	9.0 (4.5-9.8)	N/A
Hepatologist	10.5 (8.5-11)	N/A
Gastroenterology trainee	8.5 (6.0-10.0)	N/A
Predominant practice		
Staff specialist	11.0 (9.0-13.0)	N/A
Visiting medical officer	8.0 (8.0-9.0)	N/A
Private practice	8.0 (6.3-9.8)	N/A
In training program	8.5 (6.0-10.0)	N/A
Highest level of education		
Bachelor degree	9.0 (8.0-10.0)	N/A
Masters	8.0 (7.0-11.0)	N/A
PhD	11.0 (7.0-14.0)	N/A
Involved in regular IBD multidisciplinary meeting	35 (45.5%)	6 (50.0%)
Yes	9.5 (8.0-11.0)	N/A
No	8.0 (6.0-10.0)	N/A

IQR: Interquartile range; N/A: Not applicable; IBD: Inflammatory bowel disease.

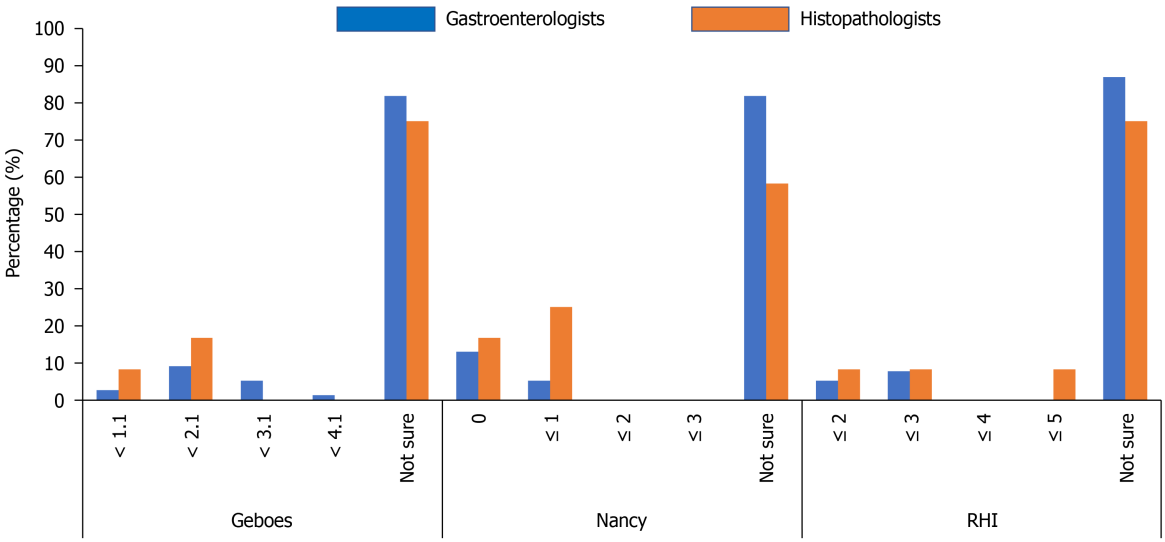
Table 5 Significant predictors of inflammatory bowel disease histology knowledge score for gastroenterologists on univariate and multivariate analyses

	Univariate analysis *P* value	Multivariate analysis *P* value
Type of subspecialty	0.005	0.03
Predominant practice	0.004	0.005
Involvement in IBD MDT	0.002	0.009
Highest level of education	0.02	

IBD: Inflammatory bowel disease; MDT: Multidisciplinary team.

would escalate medical therapy. However, when histological activity coincides with elevated faecal calprotectin, 30% were prepared to escalate treatment. These decisions match the current STRIDE-II guidelines given that histological activity is not currently an accepted target, but shows that gastroenterologists are prepared to include this endpoint as a treatment target[2]. Histological remission becomes even more important if a patient with UC had other risk factors for colon cancer, with 72% prepared to escalate treatment, given that histological activity increases the risk of colorectal neoplasia (odds ratio 3.0, 95%CI: 1.4-6.3)[5]. Therefore, when UC subjects have greater colonic disease extent, more prolonged duration of UC, presence of primary sclerosing cholangitis, or presence of a family history of colorectal cancer, gastroenterologists might escalate treatment in the presence of histological disease activity irrespective of symptoms.

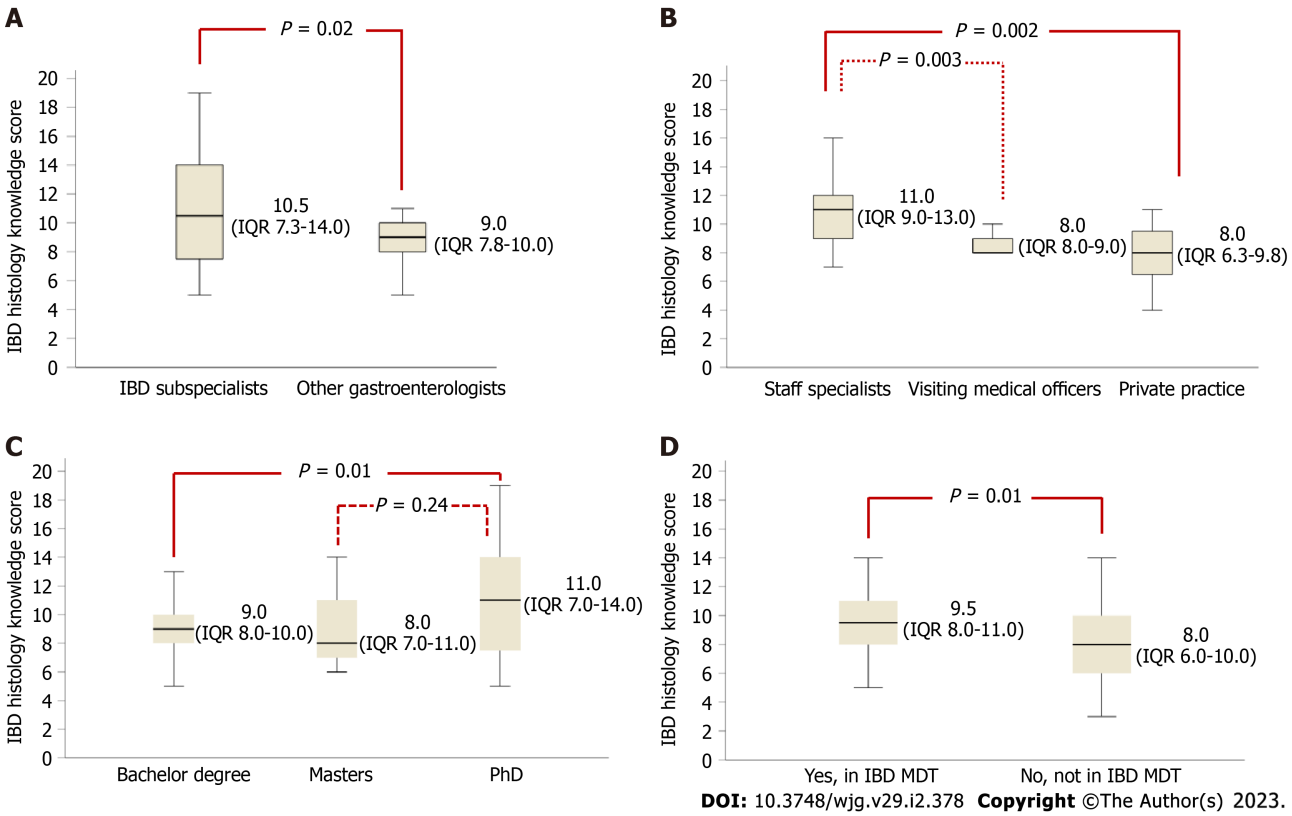
Despite the awareness of the importance of histology in UC, our survey demonstrated a lack of knowledge of histological scoring systems by gastroenterologists. Clinical trials have used Nancy index, RHI and the Geboes score but recent European Crohn's and Colitis Organisation (ECCO) guidelines recommended the use of the Nancy index and RHI for randomised clinical trials, and the Nancy index for clinical practice given its ease of use[14]. Gastroenterologists did not know which scoring systems



Percentage of gastroenterologists	2.6	9.1	5.2	1.3	81.8	13	5.2	0	0	81.8	5.2	7.8	0	0	87
Percentage of histopathologists	8.3	16.7	0	0	75	16.7	25	0	0	58.3	8.3	8.3	0	8.3	75

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Figure 2 Knowledge of histological remission definitions for scoring systems by gastroenterologists and pathologists. RHI: Robarts histopathology index.



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Figure 3 Comparisons of inflammatory bowel disease histology knowledge score for gastroenterologists. A: Subspecialty type; B: Predominant practice; C: Highest education level; and D: Involvement in inflammatory bowel disease multidisciplinary team. IBD: Inflammatory bowel disease; IQR: Interquartile range; MDT: Multidisciplinary team.

had undergone the most validation, or were unaware of the histological remission scores for the Geboes score (91%), Nancy index (87%) and RHI (92%). Despite the increasing interest and evolving role of histological scoring systems in UC, there is an opportunity to educate gastroenterologists about these

scoring systems and how to apply them in clinical practice. Predictors for higher knowledge included employment as a public hospital staff specialist and involvement in an IBD multidisciplinary team. As such, it is likely working in public hospitals within an IBD team would lead to increased exposure to the understanding of common histological scoring systems in UC. Conversely, gastroenterologists working in private practice would have less exposure to these scoring systems and their utility in UC management, contributing to lower knowledge scores.

Few studies have evaluated pathologists' views on histological activity, but most believe that they have a role in evaluating UC. However, pathologists' knowledge of UC histology was comparable to gastroenterologists [median knowledge score 8.0 (IQR: 6.5-10.0) *vs* 9.0 (IQR: 7.8-11.0) $P = 0.54$]. Similar to gastroenterologists, they also lacked knowledge of histological scoring systems and their remission definitions. There is an opportunity, therefore, to improve the utilisation of histological activity scoring for both pathologists and gastroenterologists. A harmonised approach to histological assessment in UC is lacking[16]. Future directions should include the development of histology consensus guidelines in consultation with pathologists to ensure homogeneity in reporting across hospitals to permit comparability of mucosal biopsies across different sites.

This study has several limitations. First, responder bias may have played a role, whereby responders having greater knowledge were more likely to take part on the survey. However, this would indicate a greater unawareness of histological activity scoring in the assessment of UC and a greater need for education and a harmonized approach towards the adoption of a scoring system. Secondly, a smaller respondent number for pathologists was surveyed. However, we demonstrated statistically that pathologists did not differ in their knowledge of histological scoring systems in UC despite expertise in reading biopsy histology. Thirdly, the results may lack worldwide generalisability given the survey was sent to Australian health professionals.

Strengths of this study included: (1) Being the first to report gastroenterologists' knowledge and attitudes towards the use of histology in UC; (2) recruitment of pathologists to compare their awareness against gastroenterologists; and (3) to target respondents nationwide to demonstrate generalisability.

CONCLUSION

The study highlights that while there is an acknowledgment of the importance of histological assessment in UC, there is a lack of knowledge of histological scoring systems. It indicates areas of educational need in the field of UC histology, and the importance of including pathologists in developing future consensus guidelines on the use of histology in clinical practice.

ARTICLE HIGHLIGHTS

Research background

The role of histology in ulcerative colitis has evolved over time. Histological activity despite endoscopic remission is associated with poorer clinical outcomes, and various histological scoring systems have been developed. However, the knowledge and attitudes towards the use of histology in the management of ulcerative colitis by gastroenterologists and pathologists is unknown.

Research motivation

Although there has been an increasing literature into the use of histology in ulcerative colitis, it is unknown whether this has translated into knowledge and use by gastroenterologists and pathologists in clinical practice.

Research objectives

The main objective was to evaluate the knowledge of histology guidelines and attitudes towards the use of histology in ulcerative colitis by gastroenterologists and pathologists.

Research methods

A prospective, cross-sectional survey of gastroenterologists and pathologists was conducted in Australia. The survey was formulated by using peer-reviewed guidelines.

Research results

Of 89 responders (77 gastroenterologists, 12 pathologists), there was almost complete acceptance that histological assessment should form part of ulcerative colitis evaluation (95% gastroenterologists, 92% pathologists). However, the majority of both groups lacked awareness of the Geboes score, Nancy index and Robarts histopathological index. Higher knowledge scores were predicted by public hospital attending gastroenterologists and involvement in an inflammatory bowel disease meeting.

Research conclusions

Histological remission is a recognised target for both gastroenterologists and pathologists. However knowledge of histological scoring systems was poor.

Research perspectives

Future research should involve the development of consensus guidelines in consultation with pathologists on the use of histology in ulcerative colitis management. This should include an agreement on a standardised scoring system to ensure homogenity in reporting across hospitals to permit comparability of biopsies.

FOOTNOTES

Author contributions: Pudipeddi A and Leong RW designed the research study. Pudipeddi A, Chetwood J, Paramsothy S and Leong RW performed the research and collected data. Pudipeddi A, Chetwood J and Leong RW analysed the data. Pudipeddi A drafted the manuscript. Fung C, Christensen B, Bryant RV and Subramaniam K edited the manuscript; All authors have read and approve the final manuscript.

Institutional review board statement: The study was approved by the Sydney Local Health District Human Research Ethics Committee (HREC CH62/6/2021-055).

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Country/Territory of origin: Australia

ORCID number: Aviv Pudipeddi 0000-0002-3664-2246; Robert V Bryant 0000-0002-8106-1280; Kavitha Subramaniam 0000-0001-9867-4094; John Chetwood 0000-0002-8947-9817; Sudarshan Paramsothy 0000-0002-9097-6028; Rupert W Leong 0000-0001-5944-3488.

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SYSTEMATIC REVIEWS

Third-line and rescue therapy for refractory *Helicobacter pylori* infection: A systematic review

Pedro Vieira de Moraes Andrade, Yan Mosca Monteiro, Ethel Zimberg Chehter

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Pedro Vieira de Moraes Andrade, Yan Mosca Monteiro, Ethel Zimberg Chehter, Department of Gastroenterology, Faculdade de Medicina do ABC, Santo André 09060-650, SP, Brazil

Corresponding author: Pedro Vieira de Moraes Andrade, Academic Research, Department of Gastroenterology, Faculdade de Medicina do ABC, Av. Lauro Gomes 2000, Santo André 09060-650, SP, Brazil. pvieira.m.andrade@gmail.com

Abstract

BACKGROUND

Due to increasing resistance rates of *Helicobacter pylori* (*H. pylori*) to different antibiotics, failures in eradication therapies are becoming more frequent. Even though eradication criteria and treatment algorithms for first-line and second-line therapy against *H. pylori* infection are well-established, there is no clear recommendation for third-line and rescue therapy in refractory *H. pylori* infection.

AIM

To perform a systematic review evaluating the efficacy and safety of rescue therapies against refractory *H. pylori* infection.

METHODS

A systematic search of available rescue treatments for refractory *H. pylori* infection was conducted on the National Library of Medicine's PubMed search platform based on Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines. Randomized or non-randomized clinical trials and observational studies evaluating the effectiveness of *H. pylori* infection rescue therapies were included.

RESULTS

Twenty-eight studies were included in the analysis of mean eradication rates as rescue therapy, and 21 of these were selected for analysis of mean eradication rate as third-line treatment. For rifabutin-, sitafloxacin-, levofloxacin-, or metronidazole-based triple-therapy as third-line treatment, mean eradication rates of 81.6% and 84.4%, 79.4% and 81.5%, 55.7% and 60.6%, and 62.0% and 63.0% were found in intention-to-treat (ITT) and per-protocol (PP) analysis, respectively. For third-line quadruple therapy, mean eradication rates of 69.2% and 72.1% were found for bismuth quadruple therapy (BQT), 88.9% and 90.9% for bismuth quadruple therapy, three-in-one, Pylera® (BQT-Pylera), and 61.3% and 64.2% for non-BQT) in ITT and PP analysis, respectively. For rifabutin-, sitafloxacin-, levofloxacin-, or metronidazole-based triple therapy as rescue therapy, mean eradication

rates of 75.4% and 78.8%, 79.4 and 81.5%, 55.7% and 60.6%, and 62.0% and 63.0% were found in ITT and PP analysis, respectively. For quadruple therapy as rescue treatment, mean eradication rates of 76.7% and 79.2% for BQT, 84.9% and 87.8% for BQT-Pylera, and 61.3% and 64.2% for non-BQT were found in ITT and PP analysis, respectively. For susceptibility-guided therapy, mean eradication rates as third-line and rescue treatment were 75.0% in ITT and 79.2% in PP analysis.

CONCLUSION
We recommend sitafloxacin-based triple therapy containing vonoprazan in regions with low macrolide resistance profile. In regions with known resistance to macrolides or unavailability of bismuth, rifabutin-based triple therapy is recommended.

Key Words: *Helicobacter pylori*; Refractory infection; Third-line therapy; Rescue therapy; Eradication; Treatment

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Core Tip: The eradication of *Helicobacter pylori* is widely discussed given the high prevalence and incidence of its infection. Even with established criteria in the V Maastricht Consensus for the eradication of infection and treatment algorithms for choosing first-line and second-line therapeutic regimens, therapeutic failure is frequent. Therefore, establishing safe, effective, and accessible third-line and rescue therapies for patients in need of eradication is necessary in the management of such infection. Due to this need, the present systematic review performed a systematic review evaluating the efficacy and safety of rescue therapies against refractory *Helicobacter pylori* infection.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative microaerophilic bacterium with a wide genomic diversity, which are the product of mutations, recombination, migrations, and genetic drift that favored the emergence of multiple populations and subpopulations of this bacterium[1-3]. *H. pylori* is a microorganism of global relevance, infecting about 50% of the world population[4].

Exclusive or multifactorial infection by *H. pylori* is associated with the onset of multiple diseases. The exclusive action of *H. pylori* through its virulence factors is related to the development of peptic ulcer, duodenal ulcer, gastritis, and consequently, dyspepsia[5-7]. Although *H. pylori* infection is often the primary cause of gastric cancers, the development of this pathological process results from a multifactorial interaction between bacterial, host, and environmental factors[8]. Furthermore, *H. pylori* can also stimulate lymphocytic infiltration in the gastric mucosa, which combined with high-risk genotypes may be associated with a neoplastic transformation into mucosa-associated lymphoid tissue lymphoma[9,10].

According to the IV Brazilian Consensus on Infection by *H. pylori* and the Maastricht V/Florence Consensus, the eradication of *H. pylori* is recommended in cases of peptic ulcer, mucosa-associated lymphoid tissue lymphoma, atrophic gastritis, after gastric cancer resection and in patients with first-degree relatives with gastric cancer. However, in addition to its adverse effects, the eradication of *H. pylori* can result in changes in the stomach, intestine, pancreas, and other systems and allow the colonization of other bacteria. Therefore, the risk/benefit ratio of this therapy must be evaluated by the physician[11,12].

Treatment of *H. pylori* infection is based on a combination of antimicrobials and antisecretory agents that promote an increase in gastric pH, enabling the action of antimicrobials. The increasing rates of *H. pylori* resistance to the classes of antimicrobials commonly used in conventional therapeutic regimens has reduced the effectiveness of these drugs, and failures in eradication therapies have become increasingly frequent. In an attempt to combat the growing resistance to antimicrobials, new therapeutic regimens have been used as an alternative to conventional regimens. The association of bismuth, the use of new classes of antisecretory agents such as the competitive inhibitor of potassium channels, and the adoption of new antimicrobials have acted as an alternative to standard therapeutic regimens.

Although the Maastricht V/Florence Consensus presents very well-established criteria for the eradication of infection and treatment algorithms for the choice of first-line and second-line therapeutic regimens (Figure 1) against *H. pylori* infection, there is no clear recommendation for third-line and rescue regimens in refractory *H. pylori* infection. Given the need to establish safe, effective, and accessible therapies for patients, the aim of this study was to evaluate the efficacy and safety of third-line and rescue therapies in refractory *H. pylori* infection.

MATERIALS AND METHODS

Although no review protocol was registered, the present review was conducted in accordance with Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2009 guideline, from a survey of available rescue treatments for refractory *H. pylori* infection in scientific articles on the PubMed search platform of the National Library of Medicine. The search was performed between April 22, 2021 and August 20, 2021. Different descriptors were used throughout the study for maximization of the database, namely: *Helicobacter pylori* multidrug resistance and rescue therapy; *H. pylori* multiresistant and rescue treatment; *Helicobacter pylori* multidrug resistance and rescue treatment; *Helicobacter pylori* rescue therapy; *Helicobacter pylori* and third line treatment; and fourth line therapy and *Helicobacter pylori* . After applying the inclusion and exclusion criteria, the selected articles were analyzed in two stages: first by two independent reviewers and later by the senior reviewer in order to minimize the possibility of errors and bias by the authors.

Information from articles selected and approved in both stages was extracted by reviewers independently to ensure reliable data detection and collection. A statistical analysis was performed from relevant data to the objective of this review to compare the results found in the studies. In addition to the analysis of eradication rates both by intention to treat (ITT) and per protocol (PP), a comparative analysis on adverse effects found in the different therapeutic approaches was also performed to assess their feasibility in clinical practice.

Due to the heterogeneity pool of objectives in the articles (most of them evaluated different classes or combinations of antibiotics), the level of evidence, grade rating, and bias analysis required in the PRISMA protocol could not be analyzed. Therefore, some items of the PRISMA checklist could not be applied. All articles selected according to our inclusion and exclusion criteria were included in this review, despite their PRISMA rating grade, evidence level, or bias. It was equally challenging to present their risk of bias, outcome level assessment, and strength of evidence, even with a two-phase analysis. Therefore, some of this information may be lacking in this review, but all articles included were analyzed in detail to minimize the inclusion of low evidence information.

Inclusion criteria

The present review included randomized or non-randomized clinical trials and observational studies that evaluated the efficacy of rescue therapies in refractory *H. pylori* infection published from 2014 onwards in the search platforms defined by the authors.

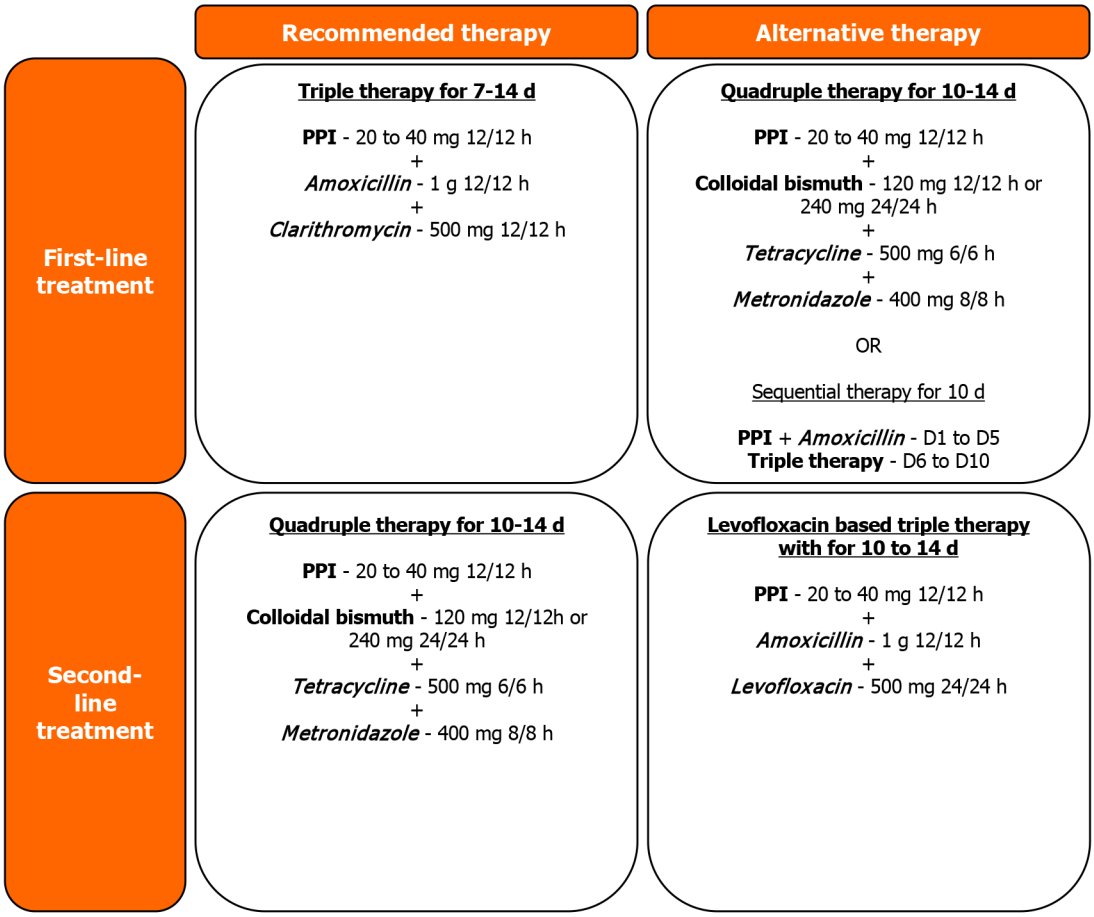
Exclusion criteria

Exclusion criteria adopted in the selection of articles of the present study were the following: Studies with pediatric patients; studies exclusively with patients who had only one failed eradication attempt; studies including patients with two or more previous failed eradication therapies, in which eradication rates for these patients were not specified; studies that did not fully discriminate the therapeutic approach used; studies without evidence of infection by *H. pylori* using methods of high sensitivity and specificity (13C-UBT and/or biopsy); and studies in which there was no subsequent follow-up of patients.

RESULTS

Selection of articles

The initial search in the PubMed database resulted in 751 potential articles. After excluding those published before January 1, 2014, 362 articles remained. After temporal delimitation and reading the abstracts of the remaining articles, 271 articles did not contain relevant information about rescue treatment. Of the 91 remaining articles containing relevant information on rescue treatment, 38 were excluded after a double check with reviewers and the senior reviewer because they were duplicates and/or statistical information related to eradication rates of third-line and rescue therapies was lacking. Articles without data on adverse effects but with eradication rates were included in this review. At the end of this stage, 53 articles were selected for analysis and read in full by the authors. Finally, 25 articles were excluded in the final stage because they did not meet the inclusion criteria in full or met any of the exclusion criteria, leaving 28 articles for inclusion. The selection process is presented in the PRISMA



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Figure 1 Therapeutic regimens recommended by Maastricht V/Florence consensus report as first-line and second-line treatment. PPI: Proton pump inhibitor.

diagram of included articles. PRISMA flow diagram reported in Figure 2.

Eradication rate

The different approaches used in the selected articles and their eradication rates can be seen in Table 1 [13-40].

Among the 28 selected articles, different active principles and therapeutic approaches were used as rescue treatment, achieving different eradication rates. Twenty-one studies were selected for analysis of the mean eradication rate as third-line treatment. Regarding the analysis of mean eradication rates of rescue therapies, studies containing patients with two or more previous failed eradications were included; the 28 studies presented in Table 1 were used. The analysis of eradication rates of regimens used as third-line treatment and rescue therapy were stratified into three subgroups based on the therapeutic regimens used, namely triple therapy, quadruple therapy, and susceptibility-guided therapy (SGT). Note that in the analysis of mean eradication rates of therapies performed in our study, therapeutic regimens were not discriminated based on the duration and dosage of the drugs used. In the absence of studies evaluating the effectiveness of therapeutic approaches as fourth-line or more, the third-line was considered as rescue therapy.

Triple therapy: Eradication rates found for triple therapy as third-line treatment were 81.6% and 84.4% for rifabutin-based regimens, 79.4% and 81.5% for sitafloxacin-based regimens, 55.7 % and 60.6% for levofloxacin-based regimens, and 62.0% and 63.0% for metronidazole-based regimen by ITT and PP, respectively (Figure 3). Regarding triple therapy as rescue treatment, mean eradication rates of 75.4% and 78.8% were found for rifabutin-based regimens, 79.4% and 81.5% for sitafloxacin-based regimens, 55.7% and 60.6% for levofloxacin-based regimens, and 62.0% and 63.0% for metronidazole-based regimen by ITT and PP, respectively (Figure 4).

Quadruple therapy: Eradication rates found for quadruple therapy as third-line treatment were 69.2% and 72.1% for bismuth quadruple therapy (BQT), 88.9% and 90.9% for bismuth quadruple therapy, three-in-one Pylera® (BQT-Pylera®), and 61.3% and 64.2% for non-BQT by ITT and PP, respectively (Figure 5). Regarding quadruple therapy as rescue treatment, mean eradication rates of 76.7% and 79.2%

Table 1 Eradication rates by therapeutic regimen

Year	Ref.	Type of study	Rescue therapy	Duration	Eradication rate
2014	Lim *et al*[13]	Randomized clinical trial	Group A: lansoprazole (30 mg, 12/12 h), amoxicillin (1 g, 8/8 h), and rifabutin 150 mg (12/12 h)	7 d	ITT: 78.1%; PP: 80.6%
2014	Lim *et al*[13]	Randomized clinical trial	Group B: lansoprazole (60 mg, 12/12 h), amoxicillin (1 g, 8/8 h), and rifabutin 150 mg (12/12 h)	7 d	ITT: 96.3%; PP: 100%
2014	Furuta *et al* [14]	Randomized clinical trial	RAS: rabeprazole (10 mg, 8/8 h or 12/12 h), amoxicillin (500 mg, 6/6 h), sitafloxacin (100 mg, 12/12 h)	7 d	ITT: 84.1%; PP: 86.4%
2014	Furuta *et al* [14]	Randomized clinical trial	RAS: rabeprazole, amoxicillin (500 mg, 6/6 h), sitafloxacin (100 mg, 12/12 h)	14 d	ITT: 88.9%; PP: 90.9%
2014	Furuta *et al* [14]	Randomized clinical trial	RMS: rabeprazole, metronidazole (250 mg, 12/12 h), sitafloxacin (100 mg, 12/12 h)	7 d	ITT: 90.9%; PP: 90.9%
2014	Furuta *et al* [14]	Randomized clinical trial	RMS: rabeprazole, metronidazole (250 mg, 12/12 h), sitafloxacin (100 mg, 12/12 h)	14 d	ITT: 87.2%; PP: 91.1%
2014	Gisbert *et al* [15]	Prospective multicenter observational study	PPI (standard dose, 12/12 h), bismuth subcitrate (120 mg 8/8 h or 240 mg, 12/12 h), tetracycline (250 mg, 6/6 h or 500 mg 8/8 h or 500 mg, 6/6 h), and metronidazole (250 mg, 8/8 h or 250 mg, 6/6 h or 500 mg, 8/8 h or 500 mg, 6/6 h)	7-14 d	ITT: 65.0%; PP: 67.0%
2014	Okimoto *et al*[16]	Randomized clinical trial	RAL: rabeprazole (10 mg, 12/12 h), amoxicillin (750 mg, 12/12 h), levofloxacin (500 mg, 24/24 h)	10 d	ITT: 45.8%; PP: 45.8%
2014	Okimoto *et al*[16]	Randomized clinical trial	RA: rabeprazole (10 mg, 6/6 h) and amoxicillin (500 mg, 6/6 h)	14 d	ITT: 40.7%; PP: 45.8%
2015	Paoluzi *et al* [17]	Randomized clinical trial	Esomeprazole (20 mg, 12/12 h), levofloxacin (500 mg, 12/12 h), doxycycline (100 mg, 12/12 h)	7 d	ITT: 40.0%; PP: 43.0%
2015	Paoluzi *et al* [17]	Randomized clinical trial	Esomeprazole (20 mg, 12/12 h), levofloxacin (500 mg, 12/12 h), doxycycline (100 mg, 12/12 h), *Lactobacillus casei* DG (24 billion units)	7 d	ITT: 54%; PP: 55%
2016	Muller *et al* [18]	Non-randomized clinical trial	Pylera® (140 mg potassium bismuth subcitrate, 125 mg metronidazole, 125 mg tetracycline, 6/6 h), omeprazole (20 mg, 12/12 h)	10 d	ITT: 83.0%; PP: 87.0%
2016	Mori *et al* [19]	Randomized clinical trial	Third-line: esomeprazole (20 mg, 6/6 h), amoxicillin (500 mg, 6/6 h), and rifabutin (300 mg, 24/24 h)	10 d	ITT: 83.3%; PP: 81.8%
2016	Mori *et al* [19]	Randomized clinical trial	Third-line: esomeprazole (20 mg, 6/6 h), amoxicillin (500 mg, 6/6 h), and rifabutin (300 mg, 24/24 h)	14 d	ITT: 94.1%; PP: 91.7%
2016	Mori *et al* [19]	Randomized clinical trial	Fourth-line: esomeprazole (20 mg, 6/6 h), amoxicillin (500 mg, 6/6 h), and rifabutin (300 mg, 24/24 h)	10 d	ITT: 77.9%; PP: 77.9%
2016	Mori *et al* [19]	Randomized clinical trial	Fourth-line: esomeprazole (20 mg, 6/6 h), amoxicillin (500 mg, 6/6 h), and rifabutin (300 mg, 24/24 h)	14 d	ITT:90.9%; PP: 90.9%
2016	Mori *et al* [20]	Randomized clinical trial	Esomeprazole (20 mg, 12/12 h), amoxicillin (500 mg, 6/6 h), and sitafloxacin (100 mg, 12/12 h)	10 d	ITT: 81.0%; PP: 82.0%
2016	Mori *et al* [20]	Randomized clinical trial	Esomeprazole (20 mg, 12/12 h), metronidazole (250 mg, 12/12 h), and sitafloxacin (100 mg, 12/12 h)	10 d	ITT: 72.4%; PP: 76.4%
2016	Chen *et al* [21]	Randomized clinical trial	Lansoprazole (30 mg, 12/12 h), potassium bismuth subcitrate (220 mg, 12/12 h), metronidazole (400 mg, 6/6 h), and amoxicillin (1 g, 8/8 h)	14 d	ITT: 88.5%; PP: 93.7%
2016	Chen *et al* [21]	Randomized clinical trial	Lansoprazole (30 mg, 12/12 h), potassium bismuth subcitrate (220 mg, 12/12 h), metronidazole (400 mg, 6/6 h), and tetracycline (500 mg, 6/6 h)	14 d	ITT: 87.2%; PP: 95.3%
2016	Noh *et al*[22]	Non-randomized clinical trial	PPI (standard dose, 12/12 h), levofloxacin (500 mg, 24/24 h), and amoxicillin (1 g, 12/12 h)	7 d	ITT: 58.3%; PP: 58.3%
2016	Noh *et al*[22]	Non-randomized clinical trial	PPI (standard dose, 12/12 h), levofloxacin (500 mg, 24/24 h), and amoxicillin (1 g, 12/12 h)	10 d	ITT: 62.5%; PP: 68.2%
2016	Noh *et al*[22]	Non-randomized clinical trial	PPI (standard dose, 12/12 h), levofloxacin (500 mg, 24/24 h), and amoxicillin (1 g, 12/12 h)	14 d	ITT: 73.7%; PP: 93.3%
2016	Hirata *et al* [23]	Non-randomized clinical trial	Esomeprazole (20 mg, 12/12 h), amoxicillin (750 mg, 12/12 h), sitafloxacin (100 mg, 12/12 h)	7 d	ITT: 83.0%; PP: 83.0%
2017	Rodríguez de Santiago *et al*[24]	Multicenter observational prospective study	Pylera® (140 mg potassium bismuth subcitrate, 125 mg metronidazole, 125 mg tetracycline, 3 capsules, 6/6 h) and esomeprazole (40 mg, 12/12 h) or omeprazole (40 mg, 12/12 h)	10 d	ITT: 80.2%; PP: 84.4%

2017	Costa *et al* [25]	Single-center observational retrospective study	SGT	-	ITT: 59.5%; PP: 61.5%
2017	Puig *et al* [26]	Multicenter observational prospective study	Esomeprazole (40 mg, 12/12 h), amoxicillin (1 g, 8/8 h), and metronidazole (500 mg, 8/8 h)	14 d	ITT: 62.0%; PP: 63.0%
2018	Fiorini *et al* [27]	Non-randomized clinical trial	Esomeprazole (40 mg, 12/12 h), amoxicillin (1 g, 12/12 h), rifabutin (150 mg, 24/24 h)	12 d	PP: 87.9%
2018	Liou *et al* [28]	Randomized clinical trial	Clinical trial 1: sequential susceptibility-guided therapy: esomeprazole (40 mg, 12/12 h) and amoxicillin (1 g, 12/12 h), for the first 7 d followed by metronidazole (500 mg, 12/12 h) and levofloxacin (250 mg, 12/12 h) or clarithromycin (500 mg, 12/12 h) or doxycycline (100 mg, 12/12 h), for another 7 d. Sequential empirical therapy: esomeprazole (40 mg, 12/12 h) and amoxicillin (1 g, 12/12 h) for the first 7 d, followed by metronidazole (500 mg, 12/12 h) and doxycycline (100 mg, 12/12 h), for another 7 d	14 d	SGT ITT: 81.0%, PP: 80.0%; Sequential empirical therapy ITT: 60.0%, PP: 60.0%
2018	Liou *et al* [28]	Randomized clinical trial	Clinical trial 2: sequential SGT: esomeprazole (40 mg, 12/12 h) and amoxicillin (1 g, 12/12 h) for the first 7 d followed by metronidazole (500 mg, 12/12 h) and levofloxacin (250 mg, 12/12 h) or clarithromycin (500 mg, 12/12 h) or tetracycline (500 mg, 12/12 h) for another 7 d. Sequential empirical therapy: esomeprazole (40 mg, 12/12 h) and amoxicillin (1 g, 12/12 h) for the first 7 d followed by metronidazole (500 mg, 12/12 h) and tetracycline (100 mg, 12/12 h) for another 7 d	14 d	SGT ITT: 78.0%, PP: 78.4%; Sequential empirical therapy ITT: 72.2%, PP: 74.4%
2018	Huang *et al* [29]	Non-randomized clinical trial	SGT: esomeprazole (40 mg, 12/12 h), amoxicillin (1 g, 12/12 h) and tetracycline (500 mg, 6/6 h) or metronidazole (500 mg, 8/8 h) or levofloxacin (500 mg, 24/24 h)	14 d	ITT: 81.4%; PP: 89.7%
2018	Huang *et al* [29]	Non-randomized clinical trial	Empirical quadruple therapy: esomeprazole (40 mg, 12/12 h), amoxicillin (1 g, 12/12 h), tetracycline (500 mg, 6/6 h), and metronidazole (500 mg, 8/8 h)	14 d	ITT: 51.8%; PP: 58.3%
2019	Saito *et al* [30]	Non-randomized clinical trial	Esomeprazole (20 mg, 12/12 h), amoxicillin (750 mg, 12/12 h), and sitafloxacin (100 mg, 12/12 h)	7 d	ITT: 54.2%; PP: 56.5%
2019	Saito *et al* [30]	Non-randomized clinical trial	Vonoprazan (20 mg, 12/12 h), amoxicillin (750 mg, 12/12 h), and sitafloxacin (100 mg, 12/12 h)	7 d	ITT: 93.0%; PP: 93.0%
2019	Sue *et al*[31]	Randomized clinical trial	Vonoprazan (20 mg, 12/12 h) amoxicillin 750 mg, (12/12 h), and sitafloxacin (100 mg, 12/12 h)	7 d	ITT: 75.8%; PP: 83.3%
2019	Sue *et al*[31]	Randomized clinical trial	Lansoprazole (30 mg, 12/12 h) or rabeprazole (10 mg, 12/12 h) or esomeprazole (20 mg, 12/12 h), amoxicillin (750 mg, 12/12 h), and sitafloxacin 100 mg, 12/12 h)	7 d	ITT: 53.3%; PP: 57.1%
2019	Ribaldone *et al*[32]	Non-randomized clinical trial	Fifth-line: rifabutin (150 mg, 12/12 h), amoxicillin (1 g, 12/12 h), and omeprazole (20 mg, 12/12 h), esomeprazole (40 mg, 12/12 h), pantoprazole (40 mg, 12/12 h) rabeprazole (40 mg, 12/12 h), or lansoprazole (30 mg, 12/12 h)	14 d	ITT: 71.5%; PP: 72.7%
2020	Liu *et al*[33]	Single center observational retrospective study	*Lactobacilli acidophilus* (1g, 8/8 h), esomeprazole (20mg, 12/12 h), potassium bismuth subcitrate (220 mg, 12/12 h), tetracycline (750 mg, 12/12 h), and furazolidone (100 mg, 12/12 h)	*Lactobacilli acidophilus* for 14 d and the others for 10 d	ITT: 92.0%; PP: 91.8%
2020	Sugimoto *et al*[34]	Single center observational retrospective study	Vonoprazan (20mg, 12/12 h), amoxicillin (500 mg, 6/6 h), and sitafloxacin (100 mg, 12/12 h)	7 d	ITT: 87.5%; PP: 87.5%
2020	Saracino *et al*[35]	Single center observational retrospective study	Third-line: esomeprazole (40 mg, 12/12 h), amoxicillin (1 g, 12/12 h), and rifabutin (150 mg, 24/24 h)	12 d	ITT: 56.1%; PP: 68.5%
2020	Saracino *et al*[35]	Single center observational retrospective study	Fourth-line: esomeprazole (40 mg, 12/12 h), amoxicillin (1 g, 12/12 h), and rifabutin (150 mg, 24/24 h)	12 d	ITT: 54.5%; PP: 63.1%
2020	Saracino *et al*[35]	Single center observational retrospective study	Fifth-line or more: esomeprazole (40 mg, 12/12 h), amoxicillin (1 g, 12/12 h), and rifabutin (150 mg, 24/24 h)	12 d	ITT: 24.4%; PP: 30.3%
2020	Saracino *et al*[35]	Single center observational retrospective	Third-line: Pylera® (140 mg potassium bismuth subcitrate, 125 mg metronidazole, 125 mg tetracycline, 6/6 h) and esomeprazole (20 mg, 12/12 h)	10 d	ITT: 87.5%; PP: 91.3%

		study			
2020	Saracino *et al*[35]	Single center observational retrospective study	Fourth-line: Pylera® (140 mg potassium bismuth subcitrate, 125 mg de metronidazole, 125 mg tetracycline, 3 capsules, 6/6 h) and esomeprazole (20 mg, 12/12 h)	10 d	ITT: 83.9%; PP: 89.6%
2020	Saracino *et al*[35]	Single center observational retrospective study	Fifth-line or more: Pylera® (140 mg potassium bismuth subcitrate, 125 mg metronidazole, 125 mg tetracycline, 3 capsules, 6/6 h) and esomeprazole (20 mg, 12/12 h)	10 d	ITT: 71.9%; PP: 74.2%
2020	Hirata *et al* [36]	Non-randomized clinical trial	Fourth-line: vonoprazan (20 mg, 12/12 h), amoxicillin (750 mg, 12/12 h), and rifabutin (150 mg, 12/12 h)	10 d	ITT: 100.0%; PP: 100.0%
2020	Ji *et al*[37]	Randomized clinical trial	Susceptibility-guided quadruple therapy: rabeprazole (10 mg, 12/12 h), colloidal bismuth (200 mg, 12/12 h), 2 sensitive antibiotics	14 d	PP: 86.49%
2020	Ji *et al*[37]	Randomized clinical trial	Rabeprazole (10 mg, 12/12 h), colloidal bismuth (200 mg, 12/12 h), amoxicillin (1 g, 12/12 h), levofloxacin (500 mg, 24/24 h), or furazolidone (100 mg, 12/12 h)	14 d	PP: 82.4%
2020	Mori *et al* [38]	Non-randomized clinical trial	Esomeprazole (20 mg, 12/12 h), amoxicillin (500 mg, 6/6 h), and sitafloxacin (100 mg, 12/12 h)	10 d	ITT: 81.6%; PP: 81.6%
2020	Nyssen *et al* [39]	Multicentric observational retrospective study	Bismuth and tetracycline-based quadruple therapy: PPI, bismuth salts (120 mg, 6/6 h or 240 mg, 12/12 h), metronidazole (500 mg, 8/8 h), and tetracycline (500 mg, 6/6 h)	10 d	ITT: 66.0%; PP: 66.0%
2020	Nyssen *et al* [39]	Multicentric observational retrospective study	Bismuth and tetracycline-based quadruple therapy: PPI, bismuth salts (120 mg, 6/6 h or 240 mg, 12/12 h), metronidazole (500 mg, 8/8 h), and tetracycline (500 mg, 6/6 h)	14 d	ITT: 82.0%; PP: 83.0%
2020	Nyssen *et al* [39]	Multicentric observational retrospective study	Bismuth and doxycycline-based quadruple therapy: PPI, bismuth salts (120 mg, 6/6 h or 240 mg, 12/12 h), metronidazole (500 mg, 8/8 h), and doxycycline (100 mg, 12/12 h)	10 d	ITT: 63.0%; PP: 63.0%
2020	Nyssen *et al* [39]	Multicentric observational retrospective study	Bismuth and doxycycline-based quadruple therapy: PPI, bismuth salts (120 mg, 6/6 h or 240 mg, 12/12 h), metronidazole (500 mg, 8/8 h), and doxycycline (100 mg, 12/12 h)	14 d	ITT: 70.0%; PP: 71.0%
2020	Nyssen *et al* [39]	Multicentric observational retrospective study	Bismuth-based quadruple therapy, three-in-one, Pylera®: PPI and Pylera®	10 d	ITT: 88.0%; PP: 88.0%
2020	Nyssen *et al* [39]	Multicentric observational retrospective study	Bismuth-based quadruple therapy, three-in-one, Pylera®: PPI and Pylera®	14 d	ITT: 100.0%; PP: 100.0%
2020	Kuo *et al*[40]	Non-randomized clinical trial	Rifabutin (150 mg, 12/12 h), amoxicillin (1 g, 12/12 h), and esomeprazole (40 mg, 12/12 h)	10 d	ITT: 77.5%; PP: 79.5%

ITT: Intention to treat; PP: Per protocol; PPI: Proton pump inhibitor; RAS: Rabeprazole, amoxicillin, and sitafloxacin; RMS: Rabeprazole, metronidazole, and sitafloxacin; SGT: Susceptibility-guided therapy.

were found for BQT, 84.9% and 87.8% for BQT-Pylera®, and 61.3% and 64.2% for non-BQT regimens by ITT and PP, respectively (Figure 6).

SGT: Eradication rates found for SGT as third-line treatment and rescue therapy were 75% and 79.2% by ITT and PP, respectively.

Adverse effects: From the reading of selected articles, information on adverse effects found in different therapeutic approaches was extracted, as shown in Table 2. The mean adverse effects rate for rifabutin-, sitafloxacin-, levofloxacin-, and metronidazole-based triple therapy in patients with two or more previous failed eradications was 53.70%, 52.36%, 13.93%, and 58.00%, respectively. With respect to adverse effects for BQT, BQT-Pylera, and non-BQT regimens, mean rates were 34.0%, 65.0%, and 45.0%, respectively. The safety of SGT was not evaluated in the present study since the choice of the therapeutic regimen was dependent on results obtained by susceptibility and genotypic resistance tests.

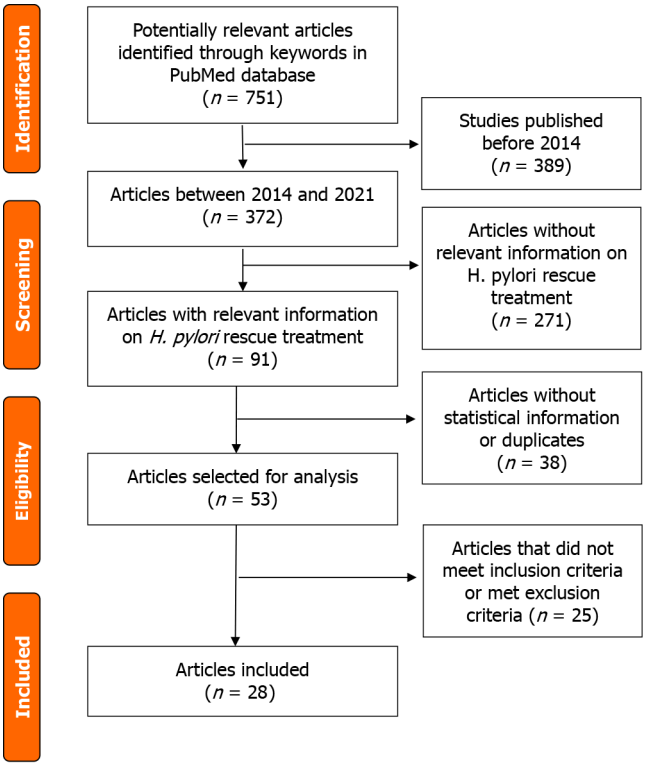
Table 2 Adverse effects

Ref.	Therapeutic scheme	Adverse effects, *n*	Total rate
Okimoto *et al* [16], 2014	Dual therapy: rabeprazole and amoxicillin	*n* = 24. Loose stools/diarrhea: 5 (20.8%); nausea: 1 (4.2%); skin rash: 0 (0%)	25%
Okimoto *et al* [16], 2014	Triple therapy: rabeprazole, amoxicillin, and levofloxacin	*n* = 24. Loose stools/diarrhea: 5 (20.8%); nausea: 0 (0%); skin rash: 1 (4.2%)	25%
Lim *et al*[13], 2014	Triple therapy: lansoprazole, amoxicillin, and rifabutin	Group A (*n* = 32). Epigastric pain: 3 (9.3%); epigastric discomfort: 2 (6.2%); asthenia: 1 (3.1%); nausea: 1 (3.1%); change in urine color: 1 (3.1%); drowsiness: 1 (3.1%); lip discomfort: 1 (3.1%); treatment was discontinued because of adverse effects: 1 (3.1%)	15.5%
Lim *et al*[13], 2014	Triple therapy: lansoprazole, amoxicillin, and rifabutin	Group B (*n* = 27). Epigastric pain: 1 (3.7); epigastric discomfort: 1 (3.7); asthenia: 0 (0%); nausea: 1 (3.7); urine color change: 0 (0%); drowsiness: 0 (0%), lip discomfort: 0 (0%)	
Furuta *et al* [14], 2014	Triple therapy: rabeprazole, sitafloxacin, and amoxicillin or metronidazole	RAS, 7 d (*n* = 44). Diarrhea: 9 (20.4%); loose stools: 20 (45.4%)	65.9%
Furuta *et al* [14], 2014	Triple therapy: rabeprazole, sitafloxacin, and amoxicillin or metronidazole	RAS, 14 d (*n* = 45). Diarrhea: 12 (26.6%); loose stools: 17 (37.7%)	64.4%
Furuta *et al* [14], 2014	Triple therapy: rabeprazole, sitafloxacin, and amoxicillin or metronidazole	RMS, 7 d (*n* = 44). Diarrhea: 8 (18.2%); loose stools: 17 (38.6%)	56.8%
Furuta *et al* [14], 2014	Triple therapy: rabeprazole, sitafloxacin, and amoxicillin or metronidazole	RMS, 14 d (*n* = 47). Diarrhea: 12 (25.5%); loose stools: 26 (55.3%)	86.3%
Paoluzi *et al* [17], 2015	Triple therapy: esomeprazole, levofloxacin, and doxycycline	*n* = 142. Swelling:; flavor perversion; mild diarrhea; treatment was discontinued because of adverse effects: 1 (0.7%)	7.7%
Mori *et al* [19], 2016	Triple therapy: esomeprazole, amoxicillin, and rifabutin	10-d group (*n* = 12). Fever: 2 (16.6%); diarrhea: 0 (0%); headache: 3 (25%); liver dysfunction: 2 (16.6%); loose stools: 2 (16.6%); urine discoloration: 1 (8.3%); allergy: 1 (8.3%); leukopenia: 1 (8.3%); stomatitis: 1 (8.3%); dysgeusia: 1 (8.3%); vertigo: 0 (0%); fatigue: 0 (0%); photophobia: 0 (0%); treatment was discontinued because of adverse effects: 1 (8.3%)	75%
Mori *et al* [19], 2016	Triple therapy: esomeprazole, amoxicillin, and rifabutin	14-d group (*n* = 17). Fever: 6 (35%); diarrhea: 5 (29.4%); headache: 3 (17.7%); liver dysfunction: 3 (17.7%); loose stools: 2 (11.8%); urine discoloration: 3 (17.7%); allergy: 2 (11.8%); leukopenia: 1 (5.9%); stomatitis: 0 (0%); dysgeusia: 0 (0%); vertigo: 1 (5.9%); fatigue: 1 (5.9%); photophobia: 1 (5.9%); treatment was discontinued because of adverse effects: 5 (29.4%)	94.1%
Mori *et al* [20], 2016	Triple therapy: esomeprazole, amoxicillin, and sitafloxacin	EAS (*n* = 63). Diarrhea: 11 (17.5%); loose stools: 9 (14.2%); constipation: 1 (1.5%); abdominal pain: 3 (4.8%); dyspepsia: 2 (3.2%); dysgeusia: 7 (11.1%); stomatitis: 3 (4.8);; allergy: 2 (3.2%); pruritus: 1 (1.5%); headache: 0 (0%); fatigue: 1 (1.5%); fever: 0 (0%); treatment was discontinued because of adverse effects: 1 (1.5%)	47.6%
Mori *et al* [20], 2016	Triple therapy: esomeprazole, metronidazole, and sitafloxacin	EMS (*n* = 58). Diarrhea: 15 (25.8%); loose stools: 8 (13.8%); constipation: 2 (3.4%); abdominal pain: 2 (3.4%); dyspepsia: 1 (1.7%); dysgeusia: 5 (8.6%); stomatitis: 2 (3.4%); ; allergy: 1 (1.7%); pruritus: 1 (1.7%); headache: 2 (3.4%); fatigue: 0; fever: 1 (1.7%); treatment was discontinued because of adverse effects: 1 (1.7%)	51.7%
Noh *et al*[22], 2016	Triple therapy: PPI, amoxicillin, and levofloxacin	-	-
Hirata *et al* [23], 2016	Triple therapy: esomeprazole, amoxicillin, and sitafloxacin	*n* = 30. Diarrhea: 5 (15.7%); rash: 1 (3.3%); asthma attack: 1 (3.3%); stomatitis: 1 (3.3%); cystitis: 1 (3.3%)	26.6%
Puig *et al*[26], 2017	Triple therapy: esomeprazole, amoxicillin, and metronidazole	*n* = 68. Diarrhea: 13 (20.0%); swelling: 3 (4.0%); dyspepsia: 14 (21.0%); taste disturbance: 23 (35.0%); nausea/vomiting: 10 (15.0%); asthenia: 4 (6.0%); others: 3 (4.0%); treatment was discontinued because of adverse effects: 2 (3.0%)	58.0%
Fiorini *et al* [27], 2018	Triple therapy: esomeprazole, amoxicillin, and rifabutin	*n* = 254. Nausea or vomiting: 6 (2.5%); abdominal pain: 13 (5.4%); mild diarrhea: 12 (5.1%); headache: 4 (1.6%); itching: 4 (1.6%); taste change: 4 (1.6%); myalgia: 1 (0.5%)	18.3%
Saito *et al* [30], 2019	Triple therapy: esomeprazole, amoxicillin, and sitafloxacin	-	-
Saito *et al* [30], 2019	Triple therapy: vonoprazan, amoxicillin, and sitafloxacin	-	-
Sue *et al*[31], 2019	Triple therapy: vonoprazan, amoxicillin, and sitafloxacin	*n* = 33. Diarrhea: 16 (50%); dysgeusia: 5 (15%); nausea: 1 (4%); anorexia: 3 (8%); abdominal pain: 5 (15%); heartburn: 6 (19%); headache: 4 (12%); eructations: 12 (35%); general malaise: 5 (16%);	-

		abdominal swelling: 12 (35%); urticaria: 3 (8%); treatment was suspended because of adverse effects: 2 (6.0%)	
Sue *et al*[31], 2019	Triple therapy: lansoprazole or rabeprazole or esomeprazole, amoxicillin, and sitafloxacin	*n* = 30. Diarrhea: 15 (51%); dysgeusia: 5 (17%); nausea: 5 (17%); anorexia: 4 (13%); abdominal pain: 6 (21%); heartburn: 4 (13%); headache: 2 (8%); eructations: 2 (8%); general malaise: 2 (8%); abdominal swelling: 6 (21%); urticaria: 2 (8%)	-
Ribaldone *et al*[32], 2019	Triple therapy: omeprazole or esomeprazole or pantoprazole or rabeprazole or lansoprazole, amoxicillin, and rifabutin	*n* = 302. Abdominal/epigastric pain: 9 (3.0%); nausea/vomiting: 7 (2.3%); diarrhea: 2 (0.7%); fatigue: 1 (0.3%); headache: 1 (0.3%); oral candidiasis: 1 (0.3%); allergy: 1 (0.3%); treatment was discontinued because of adverse effects: 4 (1.3%)	7.3%
Sugimoto *et al*[34], 2020	Triple therapy: vonoprazan, amoxicillin, and sitafloxacin	*n* = 32. Diarrhea: 4 (12.5%); loose stools: 2 (6.2%); abdominal pain: 2 (6.2%); allergic reaction: 0 (0%); others: 1 (3.1%)	28.1%
Saracino *et al*[35], 2020	Triple therapy: esomeprazole, amoxicillin, and rifabutin	*n* = 270. Diarrhea: 21 (9.3%); abdominal pain: 20 (8.8%); nausea: 17 (7.7%); headache: 15 (6.6%); dyspepsia: 14 (6.0%); treatment was discontinued because of adverse effects: 3 (1.3%)	46.4%
Saracino *et al*[35], 2020	BQT-Pylera: Pylera® and esomeprazole	*n* = 153. Nausea: 43 (29.7); drowsiness: 35 (24.1%); asthenia: 33 (22.8%); dyspepsia: 28 (19.3%); diarrhea: 26 (17.9%); treatment was discontinued because of adverse effects: 8 (5.2%)	65.5%
Hirata *et al*[36], 2020	Triple therapy: vonoprazan, amoxicillin, and rifabutin	*n* = 19. Diarrhea: 4 (21.0%); headache: 1 (5.2%); taste change: 1 (5.2%); ear fullness: 1 (5.2%)	42.0%
Gisbert *et al*[15], 2014	BQT: Bismuth, PPI, tetracycline, and metronidazole	*n* = 192. Nausea: 24 (12%); abdominal pain: 22 (11%); metallic taste: 17 (8.5%); diarrhea: 16 (8%); asthenia: 15 (7.5%); vomiting: 13 (6.5%); headache: 2 (1%); oral injury: 1 (0.5%); dizziness: 1 (0.5%); myalgia: 1 (0.5%)	22.0%
Chen *et al*[21], 2016	BQT: bismuth, lansoprazole, metronidazole, and amoxicillin	*n* = 156. Flavor distortion: 2 (1.3%); dyspepsia: 2 (1.3%); nausea: 30 (19.2%); vomiting: 4 (2.6%); abdominal pain: 1 (0.7%); swelling: 8 (5.1%); diarrhea: 1 (0.7%); dizziness: 10 (6.4%); headache: 2 (1.3%); skin rash: 3 (1.9%); fatigue: 2 (1.3%); fever: 1 (0.7%); treatment was discontinued because of adverse effects: 8 (5.2%)	34.0%
Rodríguez de Santiago *et al*[24], 2017	BQT-Pylera: Pylera® and esomeprazole or omeprazole	*n* = 101. Dyspepsia: 43 (43.9%); asthenia: 35 (35.7%); dysgeusia: 34 (34.7%); nausea: 26 (26.5%); abdominal pain: 25 (25.5%); abdominal swelling: 20 (20.4%); hyporexia: 19 (19.4%); diarrhea: 14 (14.3%); headache: 13 (13.3%); myalgia: 13 (13.3%); heartburn: 7 (7.1%); flatulence: 8 (8.1%); urticaria/eczema: 5 (5.1%); paresthesia: 4 (4.1%); arthralgia: 4 (4.1%); drowsiness: 3 (3.1%); cough: 3 (3.1%); depression: 3 (3.1%); oral aphthous ulcers: 2 (2.7%); itching: 2 (2.7%); mucous candidiasis: 2 (2.7%); insomnia: 1 (1.4%); constipation: 1 (1.4%); hypertensive crisis: 1 (1.4%)	67.3%
Huang *et al*[29], 2018	N-BQT: esomeprazole, amoxicillin, tetracycline, and metronidazole	*n* = 24. Abdominal pain: 3 (12.5%); nausea/vomiting: 3 (12.5%); constipation: 1 (4.2%); dizziness: 1 (4.2%); headache: 1 (4.2%); skin rash: 0 (0%); diarrhea: 0 (0%)	29.2%
Huang *et al*[29], 2018	Susceptibility-guided therapy	*n* = 39, Abdominal pain: 3 (7.7%); nausea/vomiting: 3 (7.7%); constipation: 2 (5.1%); dizziness: 0 (0%); headache: 1 (2.6%); skin rash: 1 (2.6%); diarrhea: 0 (0%)	25.6%
Liu *et al*[33], 2020	BQT: bismuth, esomeprazole, tetracycline, furazolidone, and *Lactobacillus acidophilus*	*n* = 50. Loose stools: 1 (2.0%); dizziness: 4 (8.0%); skin rash: 2 (4.0%); foot joint pain: 1 (2.0%); dry mouth: 3 (6.0%)	20.0%
Ji *et al*[37], 2020	BQT: bismuth, rabeprazole, amoxicillin, levofloxacin or furazolidone	*n* = 191. Abdominal pain: 4 (2.09%); constipation: 2 (1.04%); nausea: 11 (5.7); diarrhea: 5 (2.6%); flatulence: 7 (3.6%); skin rash: 5 (2.6%); pruritus: 1 (0.5%); dysgeusia: 1 (0.5%); headache: 3 (1.6%); anorexia: 0 (0%); dizziness: 8 (4.1%); dyspepsia: 6 (3.1%); drowsiness: 0 (0%); Fever: 2 (1.0%); paresthesia: 1 (0.5%); tachycardia: 2 (1.0%); vomiting: 1 (0.5%); fatigue: 2 (1.0%); suspended treatment because of adverse effects: 6 (3.1%)	20.4%
Ji *et al*[37], 2020	Susceptibility-guided therapy	*n* = 163. Abdominal pain: 8 (4.9%); constipation: 2 (1.2%); nausea: 10 (6.2%); diarrhea: 3 (1.8%); flatulence: 9 (5.5%); skin rash: 2 (1.2%); pruritus: 3 (1.8%); dysgeusia: 6 (3.7%); headache: 2 (1.2%); anorexia: 1 (0.6%); dizziness: 3 (1.8%); dyspepsia: 1 (0.6%); drowsiness: 1 (0.6%); fever: 1 (0.6%); paresthesia: 0 (0%); tachycardia: 0 (0%); vomiting: 0 (0%); fatigue: 0 (0%); treatment was discontinued because of adverse effects: 2 (1.2%)	23.3%
Nyssen *et al*[39], 2020	BQT-Pylera: Pylera® and PPI	*n* = 275. Nausea: 45 (16.0%); metallic taste: 13 (4.7%); diarrhea: 44 (16.0%); vomiting: 27 (9.8%); fatigue: 33 (12.0%); abdominal pain: 22 (8.0%); anorexia: 32 (12.0%)	42.0%
Nyssen *et al*[39], 2020	BQT: bismuth, PPI, metronidazole, and tetracycline	*n* = 85. nausea: 35 (41.0%); metallic taste: 30 (35.0%); diarrhea: 22 (26.0%); vomiting: 15 (18.0%); fatigue: 10 (12.0%); abdominal pain: 5 (5.9%); anorexia: 6 (7.1%)	52.0%
Nyssen *et al*[39], 2020	BQT: bismuth, PPI, metronidazole, and doxycycline	*n* = 94. nausea: 12 (13.0%); metallic taste: 5 (5.3%); diarrhea: 3 (3.2%); vomiting: 3 (3.2%); fatigue: 4 (4.3%); abdominal pain: 4 (4.3%); anorexia: 0 (0%)	30.0%
Costa *et al*[25], 2017	Susceptibility-guided therapy	*n* = 42. Abdominal pain: 7 (16.7%); diarrhea: 5 (11.9%); nausea: 4 (9.5%); vomiting: 3 (7.1%); change in taste sensation: 1 (2.3%); treatment was discontinued because of adverse effects: 2 (4.7%)	35.7%
Liou *et al*[28], 2018	Susceptibility-guided therapy	Clinical Trial 1 (*n* = 21). Rash: 0 (0%); dizziness: 4 (19.0%); headache: 1 (4.8%); taste distortion: 0 (0%); swelling: 1 (4.8%); abdominal pain: 0 (0%); nausea: 1 (4.8%); vomiting: 0 (0.0%); diarrhea: 2 (9.5%); constipation: 0 (0.0%)	42.9%

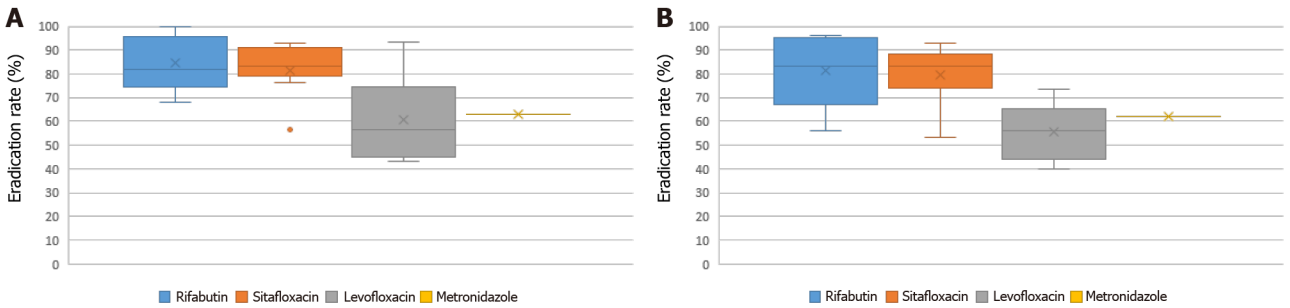
Liou *et al*[28], 2018	Susceptibility-guided therapy	Clinical Trial 2 (n = 202). Skin rash: 5 (2.5%); dizziness: 25 (12.4%); headache: 8 (4.0%); taste distortion: 7 (3.5%); swelling: 22 (10.9%); abdominal pain: 9 (4.5%); nausea: 38 (18.8%); vomiting: 14 (6.9%); diarrhea: 4 (2.0%); constipation: 1 (0.5%)	51.0%
Liou *et al*[28], 2018	N-BQT: esomeprazole, amoxicillin, metronidazole, and tetracycline	Clinical Trial 2 (n = 202). Skin rash: 3 (1.5%); dizziness: 18 (8.9%); headache: 11 (5.5%); taste distortion: 9 (4.5%); swelling: 11 (5.5%); abdominal pain: 6 (3.0%); nausea: 30 (14.9%); vomiting: 6 (3.0%); diarrhea: 14 (6.9%); constipation: 4 (2.0%)	50.5%
Liou *et al*[28], 2018	N-BQT: esomeprazole, amoxicillin, metronidazole, and doxycycline	Clinical Trial 1 (n = 20). Rash: 0 (0%); dizziness: 3 (15.0%); headache: 2 (10.0%); taste distortion: 1 (5.0%); swelling: 0 (0%); abdominal pain: 2 (10.0%); nausea: 1 (5.0%); vomiting: 0 (0%); diarrhea: 3 (15.0%); constipation: 1 (5.0%)	55.0%

BQT: Bismuth quadruple therapy; EAS: Esomeprazole, amoxicillin, and sitafloxacin; EMS: Esomeprazole, metronidazole, and sitafloxacin; N-BQT: Non-bismuth quadruple therapy; PPI: Proton pump inhibitor; RAS: Rabeprazole, amoxicillin, and sitafloxacin; RMS: Rabeprazole, metronidazole, and sitafloxacin.



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Figure 2 Preferred Reporting Items for Systematic reviews and Meta-Analyzes flow diagram. *H. pylori*: *Helicobacter pylori*.

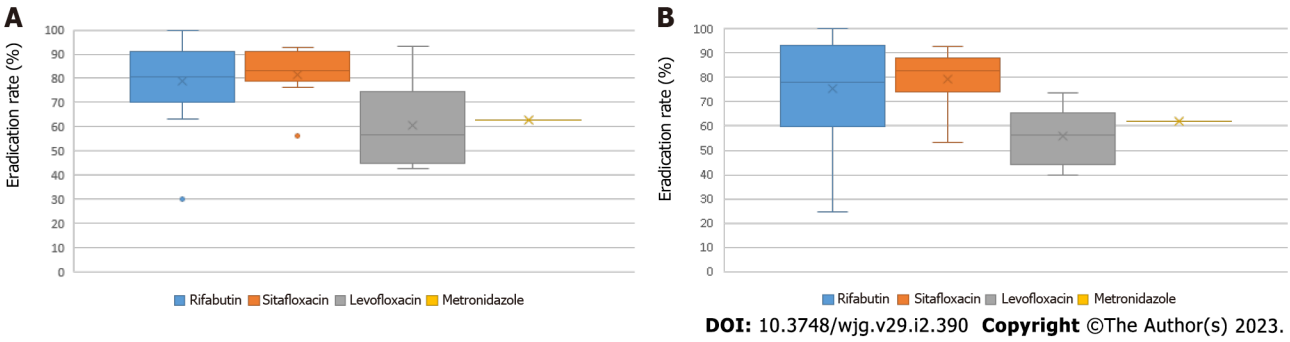


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Figure 3 Third-line triple therapy eradication rates. A: Triple therapy as third line (per protocol); B: Triple therapy as third line (intention to treat).

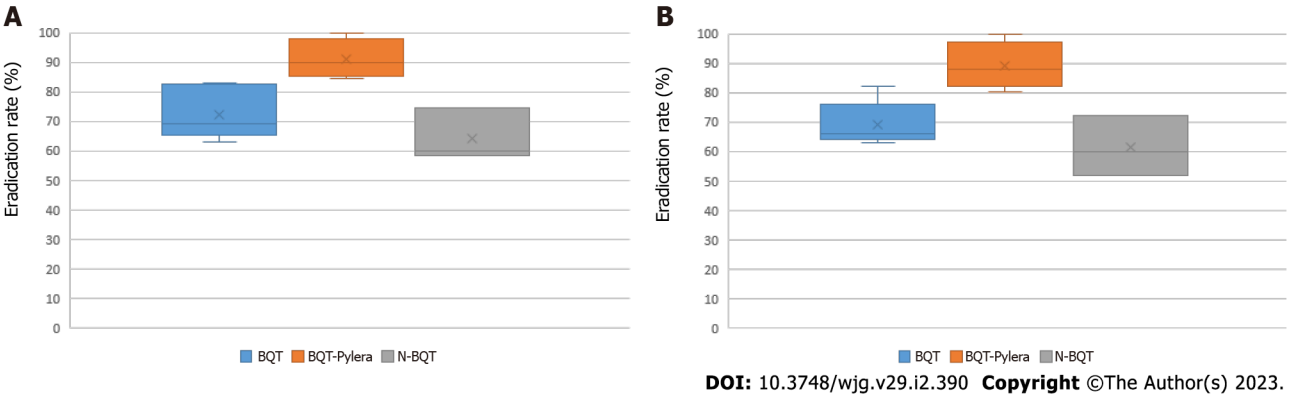
DISCUSSION

Given the high prevalence and incidence of *H. pylori* infection, its eradication is widely discussed in the



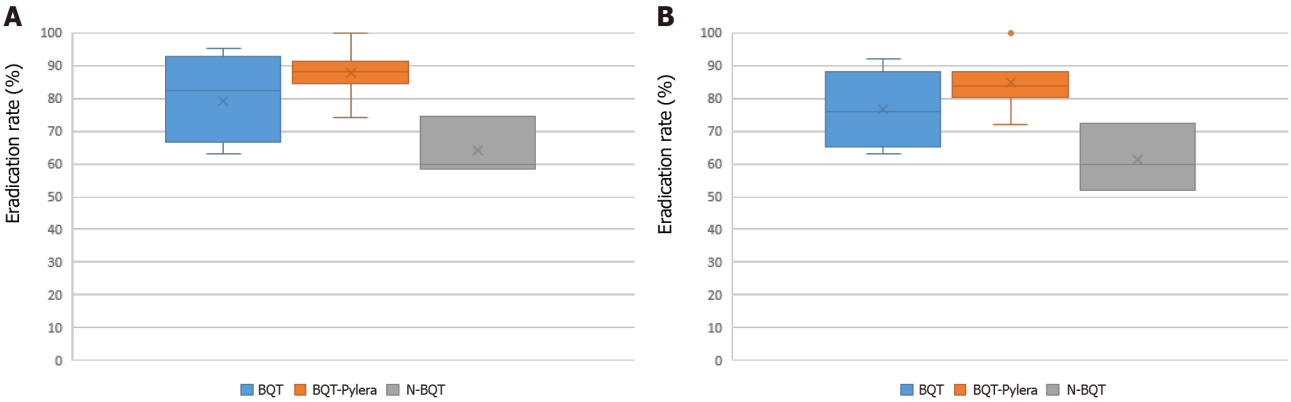
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Figure 4 Triple therapy eradication rates as rescue treatment. A: Triple therapy as rescue treatment (per protocol); B: Triple therapy as rescue treatment (intention to treat).



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Figure 5 Third-line quadruple therapy eradication rates. A: Quadruple therapy as third-line treatment (per protocol); B: Quadruple therapy as third-line treatment (intention to treat). BQT: Bismuth quadruple therapy; N-BQT: Non-bismuth quadruple therapy.



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Figure 6 Quadruple therapy eradication rates as rescue treatment. A: Quadruple therapy as rescue treatment (per protocol); B: Quadruple therapy as rescue treatment (intention to treat). BQT: Bismuth quadruple therapy; N-BQT: Non-bismuth quadruple therapy.

current scenario. Even with very well-established criteria for eradicating the infection and treatment algorithms for choosing first-line and second-line regimens against *H. pylori* infection, therapeutic failure is still very frequent. Possible causes responsible for failure to eradicate *H. pylori* include factors related to the microorganism, host, or the treatment itself, such as poor adherence of patients because of adverse effects and complexity of therapeutic regimens[41-43]. Thus, it is necessary to establish safe, effective, and accessible third-line and rescue therapies for patients.

The Maastricht V/Florence Consensus states that after failure of a first-line therapy containing clarithromycin and BQT second-line, SGT or an empirical therapy based on fluoroquinolones should be used or a combination of different antibiotics with bismuth in regions with a profile of known fluoroquinolone resistance. In cases of failure of first-line treatment based on triple or quadruple

therapy without bismuth and second-line treatment containing fluoroquinolones, the use of BQT as third-line is recommended. After failure to use BQT as first-line and therapy containing fluoroquinolones as second-line, the use of clarithromycin-based triple therapy or quadruple therapy is recommended. However, given the low level of evidence and recommendation of all these statements, their incorporation in clinical practice is difficult[12].

Triple therapy

Rifabutin: Rifabutin-based triple therapy regimens have been widely discussed as an alternative for the rescue treatment of *H. pylori* infection. In the present review, most rifabutin-based triple therapy regimens used rifabutin 300 mg (150 mg twice daily or 300 mg once daily) plus amoxicillin (variable daily dosage) and a proton pump inhibitor (PPI) (variable daily dosage) lasting 7-14 d. The mean overall eradication rate of these third-line regimens was 81.6% and 84.4% by ITT and PP, respectively. Regarding the use of rifabutin-based triple therapy as a rescue regimen, *i.e.* in patients with two or more previous failed eradications, the mean overall eradication rate was 75.4% and 78.8% by ITT and PP, respectively.

In the prospective study conducted by Lim *et al*[13], the effectiveness of rifabutin-based triple therapy was evaluated according to the PPI dosage. In this study, patients who received a rifabutin-based triple therapy regimen with higher doses of PPIs had eradication rates of 96.3% and 100% by ITT and PP, respectively, whereas patients who received standard dose PPIs showed eradication rates of 78.1% and 80.6% by ITT and PP, respectively. In turn, Mori *et al*[19] performed a comparative analysis between the duration of rifabutin-based triple therapy regimens. In this study, longer duration regimens had higher eradication rates compared to shorter duration regimens, and eradication ranged from 83.3% to 94.1% and from 81.8% to 91.7% by ITT and PP, respectively. Both studies were in line with the review performed by Gisbert *et al*[42], which suggested increasing the dose of PPIs and the duration of the therapeutic regimen as a strategy for optimizing rifabutin-based treatment.

On the other hand, in studies conducted by Ribaldone *et al*[32] and Saracino *et al*[35], lower eradication rates than those of the other studies included in the present review were found, with values of 71.5% and 68.5% by ITT and 72.7% and 56.1% by PP, respectively. These results corroborate the mean eradication rate found in the same study conducted by Gisbert *et al*[42] in 2020, in which, based on an analysis of 678 patients using rifabutin-based triple therapy, an eradication rate of 69% was found for this regimen as third-line treatment. Regarding rifabutin-based triple therapy as fourth-line treatment, in the prospective study by Hirata *et al*[36] from 2020, an association between amoxicillin, rifabutin, and vonoprazan (a competitive inhibitor of potassium) was used in patients who used sitafloxacin-based third-line. The eradication rate found by Hirata *et al*[36] was 100% by ITT and PP.

Regarding adverse effects related to rifabutin-based triple therapy, the literature presents controversial consequences of this regimen. In our review, an average of 53.7% of patients using this approach had at least one adverse effect. Although most cases are related to mild and transient adverse effects, such as gastrointestinal discomfort, there is a lot of divergence between studies. In therapies with prolonged use of rifabutin, for example, Mori *et al*[19] reported a high rate of adverse effects, with 94.1% of patients having at least one effect and discontinuation of treatment by 29.4% of patients. On the other hand, Ribaldone *et al*[32] reported that only 7.3% of patients had at least one adverse effect, and treatment was discontinued by 1.3% of patients. In addition, the use of rifabutin is associated with serious adverse effects such as myelotoxicity[41]. However, only one of the studies included in this review[19] presented patients with myelotoxicity, and 6.8% of patients had transient leukopenia with recovery of hematological patterns after 1 wk of treatment.

The mean eradication rates of rifabutin-based triple therapies found in the present review are encouraging. However, the heterogeneity of studies, whether related to eradication rates or adverse effects, makes it difficult to assess the real efficacy and safety of using rifabutin-based triple therapy as third-line treatment and rescue regimen. In addition, rifabutin is used mainly for the treatment of tuberculosis and other mycobacteria, especially in the context of immunodeficiency or HIV infection, and a possible acquisition of resistance to rifabutin is a limitation to its widespread use. Resistance to rifabutin has been reported in patients with low CD4 lymphocyte counts and when intermittent dosages were used[44]. Although the use of rifabutin in the management of refractory *H. pylori* infection involves a risk, rifabutin-based therapies act as an important alternative for third-line treatment and rescue regimens, especially in regions of previously known resistance to quinolones.

Sitafloxacin: Sitafloxacin is a quinolone with low minimum inhibitory concentration for *H. pylori* that has been used as rescue therapy[14]. In the present study, most sitafloxacin-based triple therapy regimens had a treatment regimen with sitafloxacin 200 mg (100 mg twice daily) plus amoxicillin (750 mg twice daily or 500 mg four times daily) or metronidazole (250 mg twice daily) and a PPI (variable daily dose) or vonoprazan (20 mg twice daily) for 7-14 d. The mean overall eradication rate of these regimens as third-line treatment was 79.4% and 81.5% by ITT and PP, respectively.

Although eradication rates in the studies included in the present review showed satisfactory results for the use of sitafloxacin-based triple therapy as third-line treatment, results were not homogeneous between studies. In a retrospective study, Saito *et al*[30] compared the efficacy of using sitafloxacin-based therapy associated with amoxicillin and esomeprazole or vonoprazan for 7 d as third-line

treatment. In this study, the eradication rate found in the sitafloxacin-esomeprazole association was 54.2% and 56.5% by ITT and PP, respectively. The same therapeutic regimen was used in two other studies showing discrepant eradication rates. While in the prospective study by Hirata *et al*[23], eradication rates of 83.0% by ITT and PP were found, in the randomized clinical trial performed by Sue *et al*[31], eradication rates were 53.3% by ITT and 57.1% by PP.

In a systematic review[45] from 2021, 12 clinical trials were analyzed. A mean eradication rate of 80.6% was found for sitafloxacin-based therapies containing PPIs or vonoprazan for a period of 7 d, corroborating the findings in the present study. Regarding the heterogeneity of studies included in our review, discrepancies may be based on the presence of bacterial strains with mutation in the *gyrA* gene. Mutations in this gene are responsible for conferring resistance to quinolones, leading to a lower eradication rate. The relevance of the *gyrA* gene mutation status in eradication rates of quinolone-containing regimens was expressed in the review by Mori *et al*[46]. Thus, it is recommended to identify the mutation in the *gyrA* gene before using regimens containing quinolones such as sitafloxacin, especially in regions of known previous resistance to quinolones.

Although equivalent therapeutic regimens between different studies present heterogeneous eradication rates, there is agreement regarding no statistically significant difference between the efficacy of the association of sitafloxacin with metronidazole or amoxicillin and between 7-d and 10-d duration regimens. In two studies, eradication rates between regimens containing sitafloxacin-amoxicillin and sitafloxacin-metronidazole as third-line treatment were compared, finding similar results. Furuta *et al*[14] found eradication rates for the use of amoxicillin and metronidazole, respectively, of 84.1% and 90.9% by ITT and 86.4% and 90.9% by PP for 7-d regimens and 88.9% and 87.2% by ITT and 90.9% and 91.1% by PP for 14-d regimens. Similarly, for a 10-d regimen, Mori *et al*[20] found eradication rates of 81% and 72.4% by ITT and 82% and 76.4% by PP for amoxicillin and metronidazole, respectively. Regarding the duration of therapeutic regimens, in the present study, eradication rates of 73.1%, 78.3%, and 88.0% by ITT and 74.8%, 80.0%, and 91.0% by PP were found in regimens of 7 d, 10 d, and 14 d, respectively. Both the results related to the duration of regimens and the results related to the association of sitafloxacin with amoxicillin or metronidazole were in agreement with data presented by Mori *et al*[46] in a review conducted in 2020. In this study, eradication rates of 82.0% and 76.4% were found for 10-d regimens containing amoxicillin or metronidazole, respectively, with no statistically significant difference between therapeutic regimens. In addition, as in the present review, no statistically significant difference was found between eradication rates of sitafloxacin-based treatments in regimens of 7-d and 10-d duration. Thus, the choice between the association of sitafloxacin with amoxicillin or metronidazole should be based on the availability of drugs, knowledge of previously used regimens, and the presence of penicillin allergy. The choice of therapeutic regimens with a 7-d duration is also recommended to obtain greater adherence to treatment.

The present review also showed that triple therapy based on sitafloxacin plus vonoprazan is more effective than regimens containing conventional PPIs. Two studies conducted in 2019 compared the efficacy between regimens containing vonoprazan and regimens containing PPIs. Among 63 patients involved in one of the studies[31], 33 used a regimen containing vonoprazan and 31 used a regimen containing PPIs, with eradication rates of 75.8% by ITT and 83.3% by PP with the use of vonoprazan and 53.3% by ITT and 57.1% by PP with the use of PPIs. The superiority of vonoprazan in relation to PPIs was also observed in the study by Saito *et al*[30], in which, among 81 patients with two previous failed therapies, 93.0% of those who used vonoprazan obtained successful eradication of *H. pylori*, while with the use of esomeprazole, eradication rates were 54.2% by ITT and 56.5% by PP. In a review[45] from 2021, a comparative analysis between therapies containing PPIs or vonoprazan was performed, finding eradication rates of 70.1% and 88.9%, respectively, demonstrating the superiority of regimens containing vonoprazan. Therefore, the association of sitafloxacin with vonoprazan is recommended for greater treatment efficacy when available.

Regarding adverse effects related to sitafloxacin-based triple therapy, an overall adverse event rate of 52.4% was found in our review. However, most adverse effects found were mild and transient gastrointestinal disorders. The intensity and duration of these adverse effects were also evaluated in two reviews[45,46] that reported mild and transient effects. Therefore, the use of sitafloxacin-based regimens as third-line treatment may act as a safe and effective alternative for the eradication of refractory *H. pylori* infection.

Levofloxacin: The mean eradication rate found for levofloxacin-based triple therapy as third-line treatment was 55.7% by ITT and 60.6% by PP. This unsatisfactory eradication rate was homogeneous among studies included in the present review, with the exception of a non-randomized clinical trial[22] that compared the efficacy of levofloxacin-based regimens with 7-d, 10-d, and 14-d duration as third-line treatment. In this clinical trial, from the use of a levofloxacin-based triple therapy for a 14-d period, an eradication rate of 73.7% by ITT and 93.3% by PP was reported. However, for 7-d and 10-d regimens, eradication rates were 58.3% and 62.5% by ITT and 58.3% and 68.2% by PP, respectively. These unsatisfactory rates were also found by Okimoto *et al*[16] in 2014 and by Paoluzi *et al*[17] in 2015 (see Table 1).

In a prospective observational study[47], 500 patients in third-line treatment were followed, reporting an eradication rate of 75.0% for levofloxacin-based triple therapy, which was different from the findings of the present review. However, this divergence can be explained by the increasing resistance to

levofloxacin, which acts as an important factor in the failure of therapeutic regimens, as demonstrated by the meta-analysis performed by Chen *et al*[21]. The overall adverse effect rate related to levofloxacin-based triple therapy found in our review was 13.9%. Most adverse effects related to levofloxacin-based triple therapy regimens reported gastrointestinal tract disturbances of mild intensity and transient nature. As in one of the studies[22] in the present review, the follow-up of treatment-related adverse effects was not performed, and it was not included in the overall mean rate of adverse effects. Therefore, the safety of this therapeutic regimen in a 14-d regimen has not been evaluated.

Although BQT is recommended as second-line treatment by most guidelines, levofloxacin-based triple therapy is proposed as a potential alternative by the Maastricht V/Florence Consensus[12]. In addition to being associated with a wide incidence of adverse effects, BQT is also difficult to use because of the availability of bismuth in different regions. The association of these factors, together with the efficacy and safety of levofloxacin-based second-line therapies demonstrated in the systematic review by Gisbert *et al*[48], allow the use of these regimens as second-line treatment in regions with no bismuth availability or in regions with previously known resistance to clarithromycin regimens. Thus, the use of levofloxacin-based triple therapy as a third-line treatment and rescue therapy is not recommended in 7-d and 10-d regimens given the possibility of its use as a second-line treatment and low treatment efficacy. In turn, 14-d regimens require randomized clinical trials for a more accurate assessment of the efficacy and safety of this regimen as third-line treatment and rescue therapy.

Quadruple therapy

BQT: In the present review, BQT regimens featured a treatment regimen with bismuth subcitrate (variable dose) plus a PPI (variable dose) and two antibiotics (amoxicillin, metronidazole, tetracycline, levofloxacin, furazolidone, and doxycycline, variable dose) with 7-14 d duration (see Table 1). The mean overall eradication rate of these third-line regimens was 69.2% by ITT and 72.1% by PP, with the mean overall rate of rescue treatment being 76.7% by ITT and 79.2% by PP.

In the multicenter observational study by Gisbert *et al*[15], the effectiveness and safety of BQT was investigated in 200 patients with two previous failed eradications with clarithromycin- and levofloxacin-based regimens. In this study, administration of a BQT regimen as third-line resulted in a common eradication rate of 65.0% by ITT and 67.0% by PP for regimens of 7 d, 10 d, and 14 d, with no increase in therapeutic efficacy with the extension of regimens. In contrast, in the study carried out by Nyssen *et al*[39], eradication rates of 66.0% by ITT and PP for a 10-d regimen and 82.0% by ITT and 83.0% by PP for a 14-d regimen were found, reporting an increase in therapeutic efficacy with prolonged regimens. An observational study by Hsu *et al*[49] reported eradication rates of 84.0% by ITT and PP for a 10-d BQT. Thus, the expansion of the effectiveness of therapeutic regimens based on their prolongation presents heterogeneous results among studies included in our review. More comparative studies should be performed with the objective of evaluating a possible optimization of regimens based on the increase in their duration.

In addition to this possible optimization of the quadruple therapy by increasing the regimen duration, the association of different antimicrobials, such as furazolidone proved to be effective, as demonstrated by Ji *et al*[37] and Liu *et al*[33] in 2020. Similarly, a non-inferiority randomized clinical trial [21] reported satisfactory and similar eradication rates between conventional BQT and an alternative BQT containing amoxicillin, although the alternative therapy reported better adherence and safety. These studies highlight the need to perform clinical trials comparing different combinations of antimicrobials in order to accurately assess the effectiveness of these regimens.

The evaluation of the efficacy of BQT regimens in our study showed heterogeneous results given the multiple antimicrobial combinations used in therapeutic regimens. The association of these different regimens, which were equivalently accounted to find the overall mean eradication rate of BQT, acts as a limitation of our study. Therefore, the use of BQT as third-line treatment and rescue therapy requires further investigation regarding the combination of antimicrobials and duration of regimens since in our review the mean eradication rates were unsatisfactory and heterogeneous.

In addition to the conventional use of BQT, the use of BQT-Pylera was also evaluated. In the present review, three-in-one quadruple therapy regimens featured a treatment regimen with Pylera® (140 mg potassium bismuth subcitrate, 125 mg metronidazole, 125 mg tetracycline, three capsules, 6/6 h) plus a PPI (variable dose) lasting 10-14 d. The mean overall eradication rate of this regimen as third-line was 88.9% and 90.9% by ITT and PP, respectively, while the mean overall rate of rescue treatment was 84.9% by ITT and 87.8% by PP.

In the study performed by Nyssen *et al*[39] in 2020, 222 patients with two previous failed eradications used BQT-Pylera for 10 d and 5 patients for 14 d, and eradication was observed in 88.0% of patients by ITT and PP, and 100% by ITT and PP, respectively. Both Rodríguez de Santiago *et al*[24] in 2017 and Saracino *et al*[35] in 2020 found similar eradication rates for the use of BQT-Pylera for a period of 10 d, with 80.2% and 87.5% by ITT and 84.4% and 91.3% by PP, respectively. Although eradication rates found in the present review are satisfactory, it is important to evaluate optimization strategies for these regimens.

The review performed by Liou *et al*[43] suggested the increase in the dose of PPIs and the prolongation of therapeutic approaches for 14-d regimens as optimization strategies for the treatment of refractory *H. pylori* infections. However, in our review, no statistical differences were observed in the

use of these strategies for BQT-Pylera. The increase in the dose of PPI used was evaluated in the study by Rodríguez de Santiago *et al*[24], and it did not report results superior to those found by Saracino *et al*[35] in a regimen of equivalent duration. Regarding the prolongation of the therapeutic approach, even though the comparative evaluation performed by Nyssen *et al*[39] was encouraging, it had only 5 patients in the 14-d regimen. Hence, further comparative studies are needed to determine the most effective duration of this treatment regimen as triple and rescue therapy.

Regarding adverse effects of BQT, an overall rate of 34.0% was found in conventional therapy and a rate of 65.0% for BQT-Pylera. Among studies included in the present review, Rodríguez de Santiago *et al*[24] reported that 97.0% of patients had at least one adverse effect, and despite the high proportion, no impact was reported on treatment adherence related to these events. Note that most adverse effects found by the studies were mild and transient gastrointestinal disorders, which did not pose a significant limitation to the use of these therapeutic approaches. Thus, the use of BQT, either conventional or BQT-Pylera, is an effective and safe alternative for its use as rescue therapy. However, BQT is recommended by consensus[11,12] as second-line after failure of a first-line containing clarithromycin or as first-line in regions with clarithromycin resistance greater than 15%. Thus, the use of this approach as third-line, despite showing encouraging rates, is limited not only by the use of these therapies as first-line or second-line but also by the limited availability of bismuth salts in multiple regions.

Non-BQT: In the present review, non-BQT regimens featured a treatment regimen with a PPI (variable dose, see Table 1) plus amoxicillin (1 g, 12/12 h), metronidazole (variable dose, see Table 1), and tetracycline (variable dose, see Table 1) or doxycycline (100 mg, 12/12 h) for 14 d. The mean overall eradication rate of these regimens as third-line was 61.3% and 64.2% by ITT and PP, respectively.

In the clinical trial by Huang *et al*[29], the use of a non-BQT was analyzed in a sequential regimen with tetracycline in 27 patients, finding an eradication rate of 51.8% and 58.3% by ITT and PP, respectively. Ineffective eradication rates were also found by Liou *et al*[28] in a clinical trial from 2018. In this study, two clinical trials comparing quadruple therapies containing tetracycline or doxycycline were performed, resulting in eradication rates of 72.2% and 60.0% by ITT and 74.4% and 60.0% by PP, respectively. Regarding the safety of this therapeutic approach, the mean rate of adverse effects of 44.90% was found and most were mild and transient gastrointestinal effects.

Our results show that the use of non-BQT is safe but ineffective as third-line treatment. As only two studies with this therapeutic regimen were included in our review and none of them evaluated the use of this therapy in patients with three or more previous failed eradications, more clinical trials are needed for a more accurate assessment of the efficacy and safety of these regimens as third-line treatment and rescue therapy.

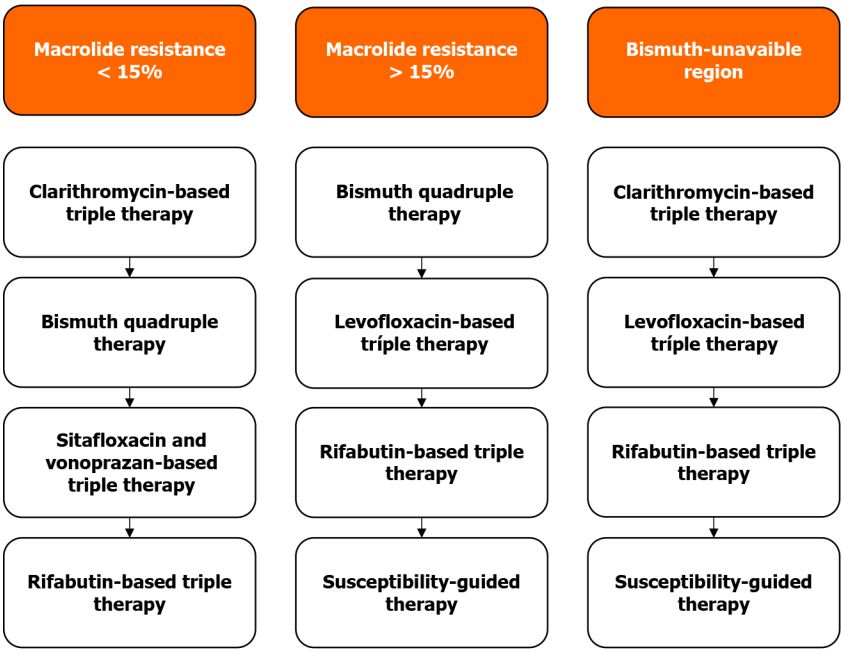
SGT

In the present review, SGT as third-line treatment had an overall mean eradication rate of 75.0% and 79.2% by ITT and PP, respectively, and these same values were found for the use of this therapy as rescue treatment. These findings, in turn, are in line with the systematic review performed by Puig *et al*[50], which found moderate results with a mean eradication rate of 72.0% by ITT and PP.

The reviews carried out by Liou *et al*[43] and Puig *et al*[50] agree with the recommendation of the Maastricht V/Florence Consensus, which suggests that SGT is recommended after failure of a second-line therapy whenever possible. However, both reviews have reservations regarding the use of this therapy as third-line treatment and as rescue therapy since the adoption of this regimen must account for the availability of tests, costs, and the patient's preference. In addition to these limitations, comparative studies between SGT and empirical regimens are limited, acting as a further obstacle to assess the practical use of this therapeutic approach as third-line and rescue therapy.

Two studies[28,37] included in the present review concluded there is no superiority in the use of SGT compared to regimens based on drug history. In the randomized clinical trial performed by Liou *et al*[28], the effectiveness of an empirical quadruple therapy and an SGT was compared in two trials. In the first clinical trial, eradication rates of 81.0% and 60.0% by ITT and 80.0% and 60.0% by PP were found for SGT regimens and empirical therapy, respectively. In the second clinical trial, eradication rates were 78.0% and 72.2% by ITT and 78.4% and 74.4% by PP, for SGT regimens and empirical therapy, respectively, concluding the non-superiority of SGT compared to empirical therapy. In contrast, the comparative clinical trial developed by Huang *et al*[29] in 2018 found the superiority of SGT in relation to empirical therapy. In this study, the eradication rate of the group that performed susceptibility tests was 81.4% and 89.7% by ITT and PP, respectively, while for the empirical group, rates were 51.8% and 58.3% by ITT and PP, respectively.

Thus, although there are not enough comparative studies to determine the real effectiveness of SGT as rescue therapy and the results presented were heterogeneous, the present review agrees with the recommendation of the Maastricht V/Florence Consensus[12]. Even if results do not show superiority in relation to empirical therapy, the use of susceptibility and genotypic resistance tests should be performed whenever possible as they provide an alternative to the growing bacterial resistance to antimicrobials.



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Figure 7 Recommendation diagram.

CONCLUSION

The present review highlighted the need to carry out a greater range of comparative studies on third-line treatment and rescue regimens in refractory *H. pylori* infection, given the increasing resistance to antimicrobials and reduction in eradication rates of therapeutic regimens. In view of recommendations of the Maastricht V/Florence Consensus, after two previous failed eradication attempts, our study also recommends performing susceptibility tests whenever possible. However, given the difficulties related to test availability, costs, and patient preference, this therapeutic approach is not always an option. Thus, the establishment of effective and safe empirical therapies is fundamental for the management of refractory *H. pylori* infection.

Among the therapeutic regimens evaluated as alternatives to third-line treatment and rescue therapy, rifabutin- or sitafloxacin-based triple therapies as well as BQT-Pylera were shown to be safe and effective. On the other hand, BQT or non-BQT and levofloxacin-based triple therapy did not present satisfactory eradication rates or presented limitations regarding their use. Therefore, although safe, their use in therapeutic management should be avoided. Note that studies related to BQT showed heterogeneous results, and further investigations regarding its use as third-line treatment and rescue therapy are necessary. Furthermore, it is also necessary to develop studies evaluating both the efficacy and the safety of regimens with levofloxacin for 14 d. As in the present review, encouraging results were found when using this regimen as third-line treatment.

From the comparison between therapeutic approaches that obtained satisfactory results as third-line treatment, the alternative with better eradication rates was the rifabutin-based triple therapy, with a mean overall eradication rate of 84.4% for third-line treatment. However, the use of rifabutin as third-line presents the risk of development of resistance by *Mycobacterium tuberculosis* as a possible limitation, and its use as third-line treatment and rescue therapy is encouraged in specific situations. Based on the encouraging results found in our study, triple therapy based on sitafloxacin containing vonoprazan is recommended as third-line treatment in regions with a low profile of macrolide resistance, and the association with amoxicillin or metronidazole should be based on availability of drugs, knowledge of previously used regimens, and the presence of allergy to penicillin since this approach had an eradication rate of at least 83.3%. Based on the promising results reported from the comparison between conventional PPIs and vonoprazan, it is important that new clinical trials are developed in order to assess the efficacy of regimens with different associations between antimicrobials and vonoprazan.

In regions with previously known resistance to macrolides or low availability of bismuth, quinolone-based therapies are used as second-line treatment, and the use of sitafloxacin-based therapies as third-line treatment and rescue therapy is not recommended. In these cases, rifabutin-based triple therapy should be used, and in cases of therapeutic failure, an evaluation of the susceptibility profile should be chosen. These recommendations can be seen in the recommendation diagram (Figure 7).

As a final consideration, despite the satisfactory mean eradication rates found with BQT-Pylera, BQTs are recommended by guidelines as second-line treatment after failure of a first-line containing clarith-

romycin or as first-line in regions with greater than 15% clarithromycin resistance, limiting its use as third-line treatment and rescue therapy. Note that the combination of three-in-one therapy drugs is related to the increase in positive outcomes in eradication, and this combination of BQT should be used instead of standardized BQTs as first- or second-line, when available.

ARTICLE HIGHLIGHTS

Research background

The eradication of *Helicobacter pylori* (*H. pylori*) is widely discussed given the high prevalence and incidence of its infection and since therapeutic failure is frequent establishing safe, effective, and accessible third-line and rescue therapies for patients in need of eradication is necessary in the management of such infection.

Research motivation

Even though eradication criteria and treatment algorithms for first-line and second-line therapy against *H. pylori* infection are well-established, there is no clear recommendation for third-line and rescue therapy in refractory *H. pylori* infection.

Research objectives

To evaluate the efficacy and safety of rescue therapies against refractory *H. pylori* infection and to establish safe, effective, and accessible third-line and rescue therapies for patients in need of eradication.

Research methods

A systematic search of available rescue treatments for refractory *H. pylori* infection was conducted on the National Library of Medicine's PubMed search platform based on Preferred Reporting Items for Systematic reviews and Meta-Analyses guidelines. Different descriptors were used throughout the study for maximization of the database, namely: *Helicobacter pylori* multidrug resistance and rescue therapy; *H. pylori* multiresistant and rescue treatment; *Helicobacter pylori* multidrug resistance and rescue treatment; *Helicobacter pylori* rescue therapy; *Helicobacter pylori* and third line treatment; and fourth line therapy and *Helicobacter pylori*. Upon reliable data detection and collection, a statistical analysis was performed to compare eradication rates both by intention to treat and per protocol, and adverse effects found in the different therapeutic approaches to assess their feasibility in clinical practice.

Research results

Twenty-eight studies were included in the analysis of mean eradication rates as rescue therapy, and 21 of these were selected for mean eradication rate analysis as third-line treatment. Rifabutin-, sitafloxacin-, levofloxacin-, and metronidazole-based triple therapies, bismuth quadruple therapy (BQT), BQT, three-in-one, Pylera® (BQT-Pylera), non-BQT, and susceptibility-guided therapy were assessed. Furthermore, sitafloxacin-based and rifabutin-based triple therapies achieved higher efficacy than other therapeutic approaches.

Research conclusions

We managed to create a recommendation flowchart regarding rescue therapies in different situations, such as regions with previously known resistance to macrolides and in areas where bismuth is unavailable. These results can aid the clinical management of the *H. pylori* infection and furthermore prevent an increase in resistance rates to different antibiotics.

Research perspectives

New clinical trials should be developed in order to assess the efficacy of regimens with different associations between antimicrobials and vonoprazan, based on the promising results reported from the comparison between conventional proton pump inhibitors and vonoprazan.

FOOTNOTES

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Country/Territory of origin: Brazil

ORCID number: Pedro Vieira de Moraes Andrade 0000-0002-8207-1587; Yan Mosca Monteiro 0000-0003-0277-2023; Ethel Zimberg Chehter 0000-0002-2409-5871.

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LETTER TO THE EDITOR

Celiac disease screening in patients with cryptogenic cirrhosis

Janaina Luz Narciso-Schiavon, Leonardo Lucca Schiavon

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Janaina Luz Narciso-Schiavon, Leonardo Lucca Schiavon, Gastroenterology Division, Internal Medicine Department, Federal University of Santa Catarina, Florianopolis 88040-900, SC, Brazil

Corresponding author: Janaina Luz Narciso-Schiavon, MD, PhD, Associate Professor, Gastroenterology Division, Internal Medicine Department, Federal University of Santa Catarina, R Maria Flora Pasewang S/N, Florianopolis 88040-900, SC, Brazil.
janaina.narciso@uol.com.br

Abstract

We write a letter to the editor commenting the article "Who to screen and how to screen for celiac disease". We discuss the present literature on cirrhosis and celiac disease (CD) and recommend screening and treating CD in individuals with cryptogenic cirrhosis.

Key Words: Celiac disease; Liver cirrhosis; Liver failure; Aspartate aminotransferase; Alanine aminotransferase

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Core Tip: We discuss reasons for recommendation of celiac disease screening in patients with cryptogenic cirrhosis.

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TO THE EDITOR

We read with interest the article by Singh *et al*[1]. Liver cirrhosis is a disease with potential morbidity, which can progress to decompensation, hepatocellular carcinoma and death. A high proportion (9.15%) of patients with cryptogenic hypertransaminasemia is affected by asymptomatic celiac disease (CD)[2]. It has been proposed that the hepatic manifestation of CD is a nonspecific chronic hepatitis[3], called by some authors celiac hepatitis[4]. A higher prevalence of CD has been demonstrated in

individuals with autoimmune hepatitis[5], and anti-actin antibodies may be present in both diseases, as they are reliable for the diagnosis of type-1 autoimmune hepatitis[6] and can also be associated with severe intestinal mucosa damage in CD patients[7]. This could support an immunological link between CD and liver injury. Despite these findings, it is not known for sure whether liver disease associated with celiac has the potential to progress to liver cirrhosis, although CD is twice as common in individuals with cirrhosis of the liver as in the general population[8,9]. In this sense, studies suggest that CD can be a cause of cryptogenic cirrhosis[10,11]. Most importantly, it has been reported that a gluten-free diet (GFD) treatment can reverse the decompensation of cirrhosis and remove the patient from liver transplantation waiting list[12-14]. Joshi *et al*[9] evaluated 84 patients with chronic liver disease, and 13% were diagnosed with CD. An improvement in liver function tests and Child-Pugh score was observed after GFD treatment. Demir *et al*[10] reported five cases of children with cryptogenic cirrhosis and CD. Treatment with GFD leaded to clinical and biochemical improvement, followed by a decrease in liver and spleen size. The most important sample was reported by Wakim-Fleming *et al*[8]. They have evaluated 204 patients with biopsy proven cirrhosis of different causes, and 2.5% were diagnosed with CD. After a GFD, patients with CD showed a return to normal levels of their celiac antibodies, small bowel biopsy and liver enzymes, and none received a liver transplant[8]. The European Society for the Study of Celiac Disease states that patients with unexplained elevation of liver enzymes should be assessed for CD and recognizes that CD can be associated with severe liver disease and even liver failure[15].

For the aforementioned reasons, and because liver cirrhosis has a high potential for morbidity and mortality, we recommend screening and treating CD in individuals with cryptogenic cirrhosis[16]. And one should consider screening for celiac antibodies in patients with decompensated cirrhosis on the liver transplantation waiting list, whatever are the mechanisms involved in the deterioration of liver function.

FOOTNOTES

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Country/Territory of origin: Brazil

ORCID number: Janaina Luz Narciso-Schiavon 0000-0002-6228-4120; Leonardo Lucca Schiavon 0000-0003-4340-6820.

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REVIEW

Salvage locoregional therapies for recurrent hepatocellular carcinoma

Cody R Criss, Mina S Makary

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Cody R Criss, Heritage College of Osteopathic Medicine, Ohio University, Athens, Ohio 45701, United States

Mina S Makary, Department of Radiology, The Ohio State University Wexner Medical Center, Columbus, Ohio 43210, United States

Corresponding author: Mina S Makary, MD, Assistant Professor, Attending Doctor, Director, Radiology, The Ohio State University Wexner Medical Center, 395 West 12th Ave, 4th Floor, Columbus, Ohio 43210, United States. mina.makary@osumc.edu

Abstract

Hepatocellular carcinoma (HCC) is the second most common cause of cancer-related death worldwide. Despite the advent of screening efforts and algorithms to stratify patients into appropriate treatment strategies, recurrence rates remain high. In contrast to first-line treatment for HCC, which relies on several factors, including clinical staging, tumor burden, and liver function, there is no consensus or general treatment recommendations for recurrent HCC (R-HCC). Locoregional therapies include a spectrum of minimally invasive liver-directed treatments which can be used as either curative or neoadjuvant therapy for HCC. Herein, we provide a comprehensive review of recent evidence using salvage loco-regional therapies for R-HCC after failed curative-intent.

Key Words: Recurrent hepatocellular carcinoma; Locoregional therapy; Transarterial chemoembolization; Transarterial embolization; Transarterial radioembolization; Ablation; Salvage therapy

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Core Tip: Management of recurrent hepatocellular carcinoma (R-HCC) includes surgical resection, systemic treatment, or locoregional therapies including ablation, transarterial chemoembolization, or radioembolization, and stereotactic body radiation therapy. In the setting of recurrence, locoregional therapies offer unique advantages over surgery for select patients. Recent investigations have also highlighted the potential of combining locoregional therapies or adding systemic retreatments for R-HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for 75%-90% of liver malignancies and is the second most common cause of cancer death worldwide[1-3]. While advancements in surveillance efforts have improved prevention and screening, incidence and mortality of HCC in recent decades have gradually increased in the United States[4,5]. Prevalence is increased in East Asia and Africa, and at-risk populations include those with cirrhosis and hepatitis B or C[4,5].

Treatment strategies for patients with HCC are tailored to tumor burden, invasiveness, and liver function, stratified using the Barcelona Clinic Liver Cancer staging (BCLC)[6]. First-line and curative treatment for HCC includes surgical resection or orthotopic liver transplantation with eligibility determined *via* the Milan criteria[5,7]. In patients with early-stage HCC who are not eligible for liver transplantation, surgical resection may be performed[6]. In patients who do not qualify as surgical candidates, the use of locoregional therapies using image-guided techniques has grown in popularity over the last several decades, providing a minimally invasive treatment approach to HCC[8,9]. Locoregional therapy is comprised of radiofrequency ablation (RFA)/ thermal microwave ablation (MVA), transarterial chemoembolization (TACE), or radioembolization (TARE), which have been commonly used neo-adjunctively to bridge or downstage patients with HCC in order to meet surgical eligibility (Figure 1)[10]. Ablation, in particular, offers a curative-intent option for nonsurgical candidates with early-stage HCC (BCLC 0/A) with a corresponding 5-year survival rate of 50%-80%[11]. Locoregional therapies provide an alternative strategy with the benefit of reduced comorbidity[12], and avoidance of complications that may worsen clinical outcomes associated with traditional surgery[13,14].

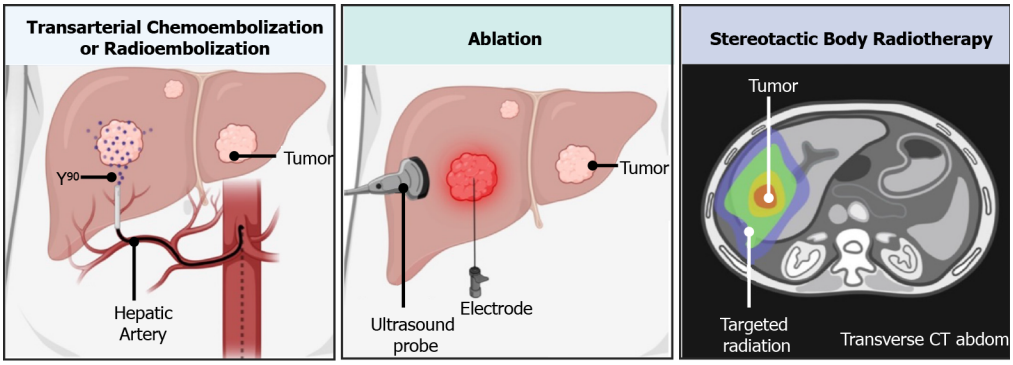
Long-term prognosis for the treatment of HCC remains poor, with a recurrence rate of 41%-70% within 5 years following resection[15-18]. Depending on tumor size, severity, liver function, and clinical indices, repeat hepatectomy may not be suitable for some patients. Therefore, alternative treatment options should be explored after initial curative attempts. No definitive consensus on standard salvage treatment approaches exist for recurrent HCC, but common therapies include repeat resection, liver transplantation, tyrosine kinase inhibitors, locoregional therapies, or a combination of multiple modalities[19]. This manuscript provides a comprehensive review of the current state of the literature for the use of salvage loco-regional therapies for recurrent HCC (R-HCC).

Risk Factors for Recurrent HCC

Prognostic factors associated with the increased risk of recurrence can vary from morphologic and surgical factors to molecular factors[20,21]. Larger tumors, or nodules with diameters ≥ 5 cm, are associated with increased rates of recurrence. Other morphological risk factors include the presence of multiple tumor nodules and satellite lesions[21-23]. The association between tumor size and recurrence is due to its correlation with invasiveness and propensity for portal vein-mediated intrahepatic metastasis and vascular invasion[21,24-26]. Microvascular invasion is a poor prognostic factor for R-HCC[27-29], defined as the histopathological observance of malignant cells within hepatic tissue and vascular cavities of the surrounding portal or hepatic vessels[30]. Other tumor-related factors associated with risk of recurrence after resection or liver transplantation, such as alpha-fetoprotein levels > 400 ug/L[31,32]. Overexpression of other histological and circulating biomarkers are also associated with negative prognostic factors related to recurrence[33].

SALVAGE LOCOREGIONAL THERAPY FOR RECURRENT HCC

Salvage locoregional therapy for R-HCC is frequently used after resection or in the setting of advanced, unresectable disease[8,9,33]. Compared to locoregional therapy or resection, liver transplantation carries a superior survival benefit for R-HCC[34-37]. However, the utility of transplantation is limited due to strict inclusion criteria, donor availability, high treatment costs, and surgical candidacy[9]. In patients who do not meet Milan criteria or not eligible for transplantation, the decision between locoregional therapies such as ablation, or repeated resection remains controversial. While resection is recognized as a primary treatment for HCC[6], portal hypertension, poor functional reserve from the future liver remnant, and technical difficulties (*e.g.*, adhesions, anatomy modifications) can make repeat resection challenging and risky[38,39]. Therefore, the efficacy of alternative methods may be uniquely promising for R-HCC. The following section includes an overview of specific locoregional therapy modalities and their efficacy for R-HCC.



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Figure 1 Schematic depiction of locoregional therapies. CT: Computed tomography.

TACE

The liver parenchyma utilizes a dual blood supply with approximately two-thirds of originating from the portal vein and the remaining third from the hepatic artery. Transarterial embolization (TAE) involves selective angiographic occlusion of tumor-supplying vessels from the hepatic artery resulting in tumor ischemia and necrosis[9,40]. Similarly, TACE involves the use of embolizing microparticles combined with regional chemotherapy[9]. Several variations of TACE exist, but embolization is commonly completed using gelatin sponge particles, polyvinyl alcohol particles, or spherical embolic agents[41]. Of note, conventional TACE utilizes a chemotherapeutic agent emulsed with lipiodol, whereas the use of drug-eluting beads carry the added benefit of increased concentration to the target[9,42,43]. Damage to healthy liver parenchyma is spared *via* arterial supply from the unobstructed portal vein[9,44].

TACE can be used as a bridge to transplantation and is currently a first-line multinodular HCC and intermediate-stage disease (BCLC B)[6,9]. It is also reserved for early-stage disease (BCLC A) who do not meet surgical criteria[9]. TACE after resection is particularly beneficial to patients with poor prognostic factors such as microvascular invasion[45-48]. Similar to primary HCC, TACE for R-HCC is tolerable and an optimal therapeutic modality for patients with poor liver function or multifocal HCC [49-51]. Two recent meta-analyses found adjuvant TACE improved overall survival (hazard ratio: 0.64-0.71)[46,52] and disease free survival (hazard ratio: 0.73)[52]. Overall 1- and 3-year survival rates for TACE for R-HCC are reportedly 28%-82% and 32%-43.9%, respectively[50,53]. Meta-analysis has reported 5-year survival rates for TACE to be 15.5%[54]. Poorer outcomes and prognosis in patients treated with TACE for R-HCC are multiple sessions, tumor size > 5 cm and ≥ 2 lesions[50]. TACE offers a unique benefit in the presence of microvascular invasion or multifocal disease but studies to date have been largely retrospective and a need for randomized control trials is required before clinical considerations are definitive. A prospective investigation of 629 patients found worse outcomes in patients treated with TACE (n = 339), compared to radiofrequency ablation (n = 162), and re-hepatectomy (n = 128)[49]. Yet, a meta-analysis of seven studies including patients with R-HCC reported no overall survival differences between TACE (n = 807) and repeated resection (n = 267). Therefore, TACE appears to be an effective treatment option for R-HCC, with a preferential advantage to patients with morphological factors such as multiple tumors or disease complicated by microvascular invasion[33].

TARE

TARE is a local radiation therapy also referred to as selective internal radiotherapy, whereby Yttrium-90 Labeled microspheres are delivered through the hepatic arteries to the tumor[55,56]. Yttrium-90 is a β-emitter, and has a tumoricidal effect at a sufficient dosage of 400Gy or greater[11]. Similar to TACE, radioembolization is used as a neoadjuvant treatment for downstaging and bridging patients for transplantation or resection[57] and considered a curative approach for early-HCC or BCLC 0/A[58]. TARE has become increasingly popular over the last decade as a safe and tolerable procedure for HCC [59], with shorter hospital length of stay and decreased risk of post-embolization syndrome when compared to TACE[60-62]. Additionally, TARE carries less risk for portal vein tumor thrombosis[63]. Recently, TARE has been adopted within the BCLC algorithm as a second-line treatment for early-stage HCC[11,64]. This change is primarily driven by the LEGACY (Local radioembolization using Glass Microspheres for the Assessment of Tumor Control with Y-90) study, which found radioembolization > 400 Gy to be safe and an effective curative approach for patients with nodules less than 8 cm[65].

For R-HCC, there is a scarcity of investigations determining the utility of TARE after failed curative-intent. Meta-analyses have shown similar outcomes between TACE and TARE for unresectable HCC [66]. It is also important to note that a randomized control trial by Salem *et al*[65] found better tumor control outcomes in patients with HCC BCLC stages A/B treated with TARE as opposed to TACE (time to progression: > 26 mo; 6.8 mo, respectively). Sangro *et al*[67] reported no differences in adverse events

in patients receiving TARE with prior failed curative-intent treatments (surgical or non-surgical) compared to treatment naïve patients receiving TARE. A retrospective investigation of 41 patients reported a time to progression of 11.3 mo and overall survival of 22.1 mo patients receiving TARE after prior resection[68]. Due to the advantages of TARE listed above, it has been advocated for advanced, unresectable disease[33,69]. More data is needed to determine the efficacy and optimal patient-selection strategies of radioembolization in the context of R-HCC.

Ablation

Ablation involves using a probe placed percutaneously under image guidance into the tumor to induce necrosis *via* thermal energy[11,70]. Ablation consists of either RFA or MVA. RFA is moderated by the "heat sink effect" which can negatively impact tumor response. Blood flow from nearby tissue can dissipate heat transfer and result in a cooling effect[71]. MVA is less impacted by heat sink due to the use of higher temperatures and larger, homogenous ablation zone, but at a cost of increased risk of injury to adjacent structures[72-75]. For both types of ablation, tumor location efficacy can be impacted by location, where tumors abutting nearby structures like the gallbladder, bowel, and diaphragm can be injured or result in insufficient safety margins that leave residual tumor[76]. Ablation is considered a curative treatment for early-stage HCC (BCLC 0/A)[6,11]. A major advantage of ablation is it can be performed quicker and may be more feasible than surgery with the added benefit of fewer complications and faster recovery[77].

A retrospective review of 211 patients with R-HCC found the 1-year survival rate for locoregional therapy (RFA, TAE, and/or percutaneous ethanol injection; n = 170, 91.6%) to be greater than salvage liver transplantation (n = 41, 90.2%)[37]. However, survival rates became superior in salvage liver transplantations at 3- and 5-years (80.4, and 80.4%, respectively) relative to the locoregional therapy group (71.7, and 51.1%, respectively)[37]. A meta-analysis of retrospective investigations by Chen *et al* [78] found improved clinical outcomes for 3- and 5-year survival rates in repeated hepatectomy compared to RFA for R-HCC. Therefore, repeated hepatectomy carries improved long-term efficacy, although the authors acknowledge selection bias may confound these results since a higher proportion of patients with improved liver function and limited tumor spread may be candidates for surgery. A meta-analysis of randomized control trials and observational studies by Yuan *et al*[79] found similar survival rates between ablation (MVI or RFA) compared to re-resection, but lower perioperative morbidity rates were observed in patients undergoing ablation (3.3%) relative to re-resection (17%). The majority of these studies included tumors ≤ 3 cm, and therefore the decision to utilize ablation over surgery for R-HCC may be appropriate for smaller tumors[80]. In tumors ≤ 3 cm, disease-free survival rates are similar to resection, but hospital length of stay and perioperative morbidity is lower in RFA (5 d, 7%, respectively) compared to repeated resection (13 d, 16%, respectively)[81]. Yang *et al*[82] echoed these findings, illustrating repeat resection for R-HCC has superior overall survival rates, but sub-group analyses of outcomes for smaller tumors diminish survival differences between these two methods. Larger, more homogenous ablation volumes associated with MVA may broaden ablation applicability to larger tumors[83]; however, studies to date evaluating MVA for R-HCC are limited.

Stereotactic body radiotherapy

Stereotactic body radiotherapy (SBRT) is a localized therapy whereby fractionated high-dose radiation is used to ablate liver parenchymal tumors (Figure 1)[84]. Conventionally, SBRT is dedicated to salvage therapy for R-HCC or advanced disease when ablation or embolization has failed or is contraindicated [85]. SBRT is currently not included in the BCLC but is included in the National Comprehensive Cancer Guidelines[84]. Kimura *et al*[86] reviewed patients with HCC who either failed or were not eligible for resection or other locoregional therapies, reporting safe and satisfactory overall survival rates for first and second SBRT (n = 81, 60.4%, and 61%, respectively). In patients receiving salvage SBRT after TACE, overall survival rates at 3 years were 72.7% (n = 302), with 95.4% tumors reaching complete response [87]. Therefore, in patients who fail TACE and curative modalities are not suitable, salvage SBRT could be offered as a potential subsequent treatment option.

Multimodal Locoregional Therapy Approaches

Approaches that combine locoregional therapies (*e.g.*, TACE and RFA/MVA) have been proposed. Several mechanisms have been suggested to explain the synergistic or additive effects of combining modalities. Multimodality therapies may overcome individual limitations of monotherapy, such as providing adequate control for intermediate to larger tumors[72,88-90]. TACE is suggested to mitigate the heat sink effect and therefore, positively impact the efficacy of RFA[71]. Chemoembolization may also reduce tumor burden, which can aid RFA by extending the safety margin and the resultant coagulation zone[90,91]. A meta-analysis of 8 randomized control trials using RFA-TACE for primary HCC found improved overall survival [hazard ratio (HR) = 0.58, confidence interval (CI) 0.41 - 0.80] and recurrence free survival (HR = 0.65 CI =0.47 - 0.76) compared to RFA alone.

To date, few investigations have sought to determine the efficacy of multimodal therapy as a salvage treatment approach in unresectable disease or instances of R-HCC. For the treatment of larger R-HCC tumors (≤ 7 cm), TACE followed by RFA can reveal additional satellite lesions and have greater 1-, 3-, 4-

year survival rates (92.6%, 66.6%, 61.8%) than RFA alone (85.3%, 59%, 45%)[92,93]. Studies comparing the efficacy of TACE-RFA have indicated comparable 1-, 3-, and 5-year survival outcomes between the two salvage treatment approaches for both smaller tumors (≤ 5 cm) [94,95] and larger ones (> 5 cm)[96]. Interestingly, TACE-RFA achieved satisfactory outcomes with a lower rate of complications (*e.g.*, bleeding, liver failure) and shorter hospital stays[94-96]. Yang *et al*[97] published a retrospective investigation of 103 patients with R-HCC treated with either RFA, TACE, or combination therapy of RFA and TACE. Intrahepatic rates of recurrence were lower in the combination group (20.7%) compared to TACE (57.1%) and the RFA group (43.2%). 1-, 3- and 5-year survival rates were also greater in the combination group (88.5%, 64.6%, 44.3%) compared to the TACE alone group (65.8%, 38.9%, 19.5%). Other multimodal regimens for R-HCC have been explored, including TACE and MVA, of which when combined, improve tumor response and prolong progression-free survival compared to TACE monotherapy for small R-HCC tumors (≤ 3 cm)[98]. Although prospective investigations are required prior to establishing recommendations, in general, current evidence indicates a potential survival benefit to multimodality approaches with some investigators advocating for the adoption of multimodal therapy in future BCLC treatment guidelines[99].

Combining Locoregional Therapy and Systemic Therapy

Sorafenib, an oral tyrosine kinase inhibitor, is reserved for advanced-stage disease (BCLC class C) based on the results of the SHARP trial[6]. Overall, Sorafenib can offer survival benefit for unresectable HCC, but worse tumor response and greater adverse events when compared to locoregional therapies[100, 101]. Challenges of using sorafenib are further compounded by heterogenous response rates and acquired resistance[102-104]. However, investigations have explored the utility of combining oral systemic agents with locoregional therapy (Table 1). A retrospective study reviewed 1126 patients with R-HCC in patients who received sorafenib and concurrent TACE or TACE monotherapy. The addition of sorafenib to TACE offered significantly improved survival time compared to TACE alone (20.23 *vs* 13.87 mo, respectively)[105]. Peng *et al*[106] retrospectively reviewed patients with advanced R-HCC receiving either sorafenib monotherapy (n = 101), or a combination of sorafenib and TACE-RFA (n = 106). While the toxicity profile was similar between both groups, median overall survival and time to progression in TACE-RFA + sorafenib (14 mo; 7 mo, respectively) was superior to sorafenib monotherapy (9 mo; 4 mo, respectively)[106]. A randomized, multicenter control trial comparing TACE (n = 76 and TACE with sorafenib (n = 80) for unresectable HCC, resection, found median progress-free survival to be greater in the combined treatment group (25.2 *vs* 13 mo)[107]. Although this trial included treatment naiive patients, a large portion of patients received prior locoregional therapy treatments Multicenter phase III randomized control trials comparing TACE alone and TACE with sorafenib for recurrent, unresectable HCC are currently underway.

FUTURE DIRECTIONS

Immuno-locoregional combination therapy

Immunological properties associated with HCC have driven a growing use of immune checkpoint modulators such as anti-PD-1 antibodies (*e.g.*, nivolumab, pembrolizumab, camrelizumab) or CTL-A-4 inhibitors (*e.g.*, ipilimumab, tremelimumab)[108-111] over the last decade. Thus far, phase 2 and 3 trials have found promising tumor response rates and safety profiles compared to previous standard systemic therapies[112]. In addition to tumor necrosis, there has been some evidence that locoregional therapy can activate T-cell responses and augment the expression of multiple immune-mediated processes within the tumor microenvironment[113]. Development of treatment strategies for HCC that combine locoregional therapies and immunomodulators have thus emerged. Despite this rise in utilization, Guo *et al*[109] found no difference in clinical outcomes or tumor response for combined TACE and camrelizumab compared to TACE monotherapy. Studies determining the efficacy of immunotherapy combined with locoregional therapy are scarce, but multiple trials combining immunomodulators and locoregional therapies are currently underway[114]. It should be noted, adverse events with immunocheckpoint blockers, such as hyperprogressive disease, have been reported and pose a unique challenge influencing clinical judgment to utilize these agents. Hyperprogressive disease is characterized by a rapid increase in tumor burden and subsequent clinical deterioration in patients treated with immunotherapy agents. Other immunotherapies benefits (*e.g.*, vaccines, oncolytic viruses and adoptive cellular therapies) have also been speculated to be therapeutic but remain under clinical investigation[111].

Determining treatment algorithms for recurrent HCC

After the failure of curative-intent or tumor recurrence, the use of locoregional therapies is warranted, especially in patients no longer eligible for surgery. Ablation, however, should be considered as a comparable alternative to repeat-resection in patients with recurrent small solitary tumors, notably ≤ 3 cm. Similar to prior reviews, in patients with early recurrence (< 1 year), multifocal disease (> 2 - 3 nodules) or in the presence of microvascular invasion, TACE should be considered[33]. Moreover, due to lower toxicity and longer time-to-progression for advanced disease[62], the use of radioembolization

Table 1 Outcomes of multimodal locoregional therapy for recurrent hepatocellular carcinoma

Ref.	Study design	Treatment	Number of patients	Outcomes
Song *et al* [95]	Retrospective	Recurrent HCC ≤ 5 cm	63 TACE; 96 TACE-RFA	TACE-RFA lower disease progression than TACE monotherapy; No difference in overall survival
Zhang *et al* [115]	Retrospective	Treatment Naïve HCC, DEB-TACE-RFA for Recurrent HCC (Group B), and hepatectomy	40 DEB-TACE as primary treatment; 36 DEB-TACE Recurrent HCC; 40 hepatectomy as primary	DEB-TACE-RFA can prolong survival time for recurrent HCC
Zheng *et al* [96]	Retrospective	TACE-RFA or repeat hepatectomy	63 TACE-RFA; 38 repeat hepatectomy	Similar overall survival for TACE-RFA (38 months) compared to repeat hepatectomy (42 months); No difference in progression free survival
Peng *et al* [94]	Retrospective	Recurrent HCC ≤ 5 cmTACE-RFA or repeat hepatectomy	107 TACE-RFA; 79 repeat hepatectomy	No difference in overall survival or disease-free survival; TACE-RFA has lower complications and shorter hospital stays
Ji *et al*[98]	Retrospective	Recurrent HCC with three or fewer tumors < 3 cm	17 TACE-MWA; 28 TACE	TACE-MWA showed better 1-,3-, 6- month tumor response; TACE-MWA showed prolonged 1-,3-, 5-year progression free survival; No difference in overall survival

HCC: Hepatocellular carcinoma; TACE: Transarterial chemoembolization; RFA: Radiofrequency ablation; DEB-TACE: Drug-eluting bead transarterial chemoembolization; MWA: Microwave ablation. TACE-RFA: Transarterial chemoembolization and radiofrequency ablation; DEB-TACE-RFA: Drug-eluting bead transarterial chemoembolization and radiofrequency ablation; TACE-MWA: Transarterial chemoembolization and Microwave ablation.

offers a favorable alternative to TACE. Evidence supports that multimodal therapy provides superior clinical benefit to monotherapy as well as repeat-resection for smaller tumors (Table 1) for R-HCC. To date, it is unclear which additional patient populations (*e.g.*, those not currently suitable for locoregional monotherapy) may benefit from multimodal or strategies that combine locoregional and systemic therapy (Table 2).

CONCLUSION

Treatment strategies for R-HCC remain a challenge, and there is no consensus on how to manage patients who fail curative-intent therapies. The use of targeted locoregional therapies can improve clinical outcomes after recurrence in patients not eligible for or awaiting transplantation, or in cases of advanced disease. The emerging use of multimodal and additive systemic agents exhibit promise as a novel treatment approach in the setting of recurrence; however, prospective studies are necessary before definitive recommendations can be made.

Table 2 Locoregional therapy and oral agents for recurrent hepatocellular carcinoma

Ref.	Study Design	Treatment	Number of Patients	Outcomes
Wan *et al*[105]	Retrospective	Recurrent HCC ≤ 5 cm	127 TACE; 127 Sorafenib + TACE	Sorafenib + TACE increased survival time compared to TACE alone (30.7 *vs* 18.22 mo); Longer duration of Sorafenib when treated with Sorafenib + TACE associated with survival
Peng *et al*[106]	Retrospective	Recurrent HCC ≤ 7 or five nodules ≤ 3 cm	106 TACE-RFA + Sorafenib; 101 Sorafenib	Longer median overall survival and time to progression for combination therapy
Guo *et al*[109]	Retrospective	Recurrent HCC	20 TACE+ camrel-izumab; 51 TACE	No difference in tumor response, progression-free survival, or overall survival

HCC: Hepatocellular carcinoma; TACE: Transarterial chemoembolization; RFA: Radiofrequency ablation; TACE-RFA: Transarterial chemoembolization and radiofrequency ablatio.

FOOTNOTES

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Country/Territory of origin: United States

ORCID number: Mina S Makary 0000-0002-2498-7132.

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REVIEW

COVID-19 and hepatic injury: cellular and molecular mechanisms in diverse liver cells

Fares E M Ali, Mostafa K Abd El-Aziz, Mahmoud M Ali, Osama M Ghogar, Adel G Bakr

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Fares E M Ali, Adel G Bakr, Department of Pharmacology and Toxicology, Faculty of Pharmacy, Al-Azhar University, Assiut 71524, Egypt

Mostafa K Abd El-Aziz, Mahmoud M Ali, Department of Pharmacology, Al-Azhar University, Assiut 71524, Egypt

Osama M Ghogar, Department of Biochemistry Faculty of Pharmacy, Badr University in Assiut, Egypt

Corresponding author: Fares E M Ali, MSc, PhD, Lecturer, Research Scientist, Department of Pharmacology and Toxicology, Faculty of Pharmacy, Al-Azhar University, Al-Azhar Street, Assiut 71524, Egypt. faresali@azhar.edu.eg

Abstract

The coronavirus disease 2019 (COVID-19) represents a global health and economic challenge. Hepatic injuries have been approved to be associated with severe acute respiratory syndrome coronavirus (SARS-CoV-2) infection. The viral tropism pattern of SARS-CoV-2 can induce hepatic injuries either by itself or by worsening the conditions of patients with hepatic diseases. Besides, other factors have been reported to play a crucial role in the pathological forms of hepatic injuries induced by SARS-CoV-2, including cytokine storm, hypoxia, endothelial cells, and even some treatments for COVID-19. On the other hand, several groups of people could be at risk of hepatic COVID-19 complications, such as pregnant women and neonates. The present review outlines and discusses the interplay between SARS-CoV-2 infection and hepatic injury, hepatic illness comorbidity, and risk factors. Besides, it is focused on the vaccination process and the role of developed vac-cines in preventing hepatic injuries due to SARS-CoV-2 infection.

Key Words: COVID-19; Hepatic injury; Viral tropism; COVID-19 comorbidity; Vaccination

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Core Tip: The association between coronavirus disease-19 and hepatic injury is demonstrated by determining the viral tropism and its different pathological implications. A better understanding of the diversity and risk factors of severe acute respiratory syndrome coronavirus-2-induced hepatic injury provides a fundamental approach to overcoming adverse effects. Moreover, vaccination can influence assessment and evaluation.

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INTRODUCTION

Coronavirus (CoV) is derived from the Latin word "corona," which means "crown"[1]. It can cause various human respiratory tract diseases, ranging from mild cold to severe respiratory distress syndrome (RDS)[2]. CoV has presented several challenges throughout its history, including viral isolation, detection, prevention, and vaccine development[3]. CoV is a member of the order *Nidovirales* and has the largest RNA genome[4]. Furthermore, it is recognized as arising from a zoonotic origin and frequently spreads by contact or respiratory droplets. The affected individual has non-specific clinical characteristics requiring virological diagnosis and molecular confirmation[5]. Seven coronaviruses have been recognized to infect humans, with SARS-CoV-2 being the most recent, and this might be due to frequent infections across different species and sporadic spillover episodes[4]. Two of these previously recognized coronaviruses are the Middle East Respiratory Syndrome CoV, which originated in the Middle East in 2012, and the Severe Acute Respiratory Syndrome CoV (SARS-CoV), which originated in China from 2002 to 2003 and was responsible for significant epidemics in the previous two decades[6]. The recent CoV illness, also known as coronavirus disease of 2019 (COVID-19), poses a risk to global health[7]. The COVID-19 pandemic began in the Chinese city of Wuhan near the end of December 2019 and spread rapidly in the following months to Thailand, Japan, South Korea, Singapore, and Iran[8]. This was followed by a viral outbreak worldwide, particularly in Spain, Italy, the United States of America, the United Arab Emirates (UAE), and the United Kingdom (UK). The COVID-19 disease is classified as a pandemic by the World Health Organization (WHO)[9]. The three types of coronaviruses are zoonotic, can infect people, and cause severe and fatal diseases[10]. New coronaviruses are expected to emerge and cause sporadic seasonal outbreaks due to their great genetic diversity, frequent genome recombination, and rise in human-animal interface activities brought on by contemporary agricultural methods[11].

VIRAL TROPISM

In COVID-19, viral tropism is responsible for spreading infection outside the respiratory tract and predisposing it to systemic symptoms, aggravating pre-existing disorders, and multiorgan damage in the kidney, heart, nervous system, liver, and gastrointestinal tract[12,13]. However, the available data indicate the second multiorgan dysfunction inherent to the immune discrepancy or cytokine storm, developing hypoxic or ischemic injury and drug-induced injury[14,15]. Although viral tropism should be considered to understand the SARS-CoV-2 infection, the S protein of the virus mediates SARS-CoV-2 cell entrance, which represents a high affinity for cells expressing angiotensin-converting enzyme 2 receptors (ACE2)[16]. Furthermore, the affinity of the S protein to ACE2 receptors increases when SARS-CoV-2 is proteolytically activated[17]. In an *in vitro* study by Letko *et al*[18], the S protein of lineage B beta-coronaviruses such as SARS-CoV and the recent SARS-CoV-2 significantly improved its affinity for its receptor when it was pre-incubated with proteolytically activated trypsin. Trypsin is expressed by liver epithelial cells[19]. Additionally, the protein of the SARS-CoV-2 contains a furin-like proteolytic site that has never been observed in other coronaviruses[20]. It is worth mentioning that furin is expressed in organs such as the salivary glands, liver, kidney, and pancreas involved in SARS-CoV-2 infection[21]. As a result, to determine tropism for a particular tissue, ACE2 should be present at the host cell surface[22]. Consequently, ACE2 expression is considered a mirror of viral load[23]. Controversially, the highest levels of ACE2 are detected in the small intestine, testis, heart, colon, and thyroid gland[24]. Nevertheless, respiratory system symptoms are dominant in COVID-19 because the nasal ciliated cells are the primary targets for SARS-CoV-2 replication in the early stages of infection[25]. Besides, ACE2 is abundantly expressed in more than 80% of alveolar lung cells, consequently affecting all respiratory functions[23].

DIAGNOSIS

With increasing COVID-19 prevalence and mortality rates, as of 14 August 2022, the WHO reported that over 587 million people were infected with SARS-COV-2, including over 6 million deaths[26]. Therefore, the nation's healthcare systems face overwhelming psychological and economic burdens. Consequently, the most efficient method to prevent infection is to separate symptomatic persons, quarantine others, and manage concomitants while increasing immunization rates.

The molecular test is the most practical method to confirm the diagnosis of COVID-19, using the reverse transcription polymerase chain reaction (RT-PCR) to detect viral genetic materials in different sample swabs from the nasal cavity, mouth, sputum, and feces[27,28]. This molecular test provides high sensitivity and specificity; however, it has several drawbacks, such as requiring trained technicians, being time-consuming, high cost, shortages in test kit supplies, and false negative thresholds[29]. Therefore, it is critical to develop new quick, reliable, and affordable diagnostic techniques.

Patients with fever, cough, and chest pain with breathing problems or pneumonia are usually diagnosed by imaging tests, such as chest X-ray or computed tomography (CT)[30]. Imaging tests are predominantly available worldwide, and the scanning process is relatively simple and rapid, enabling a large population's screening[31]. In a study based on chest X-ray findings and severity scores, a chest X-ray is a limited tool because it has an abnormality observed at a specific point[32]. In the same context, Borghesi A. and R. Maroldi mentioned that chest X-ray is an insensitive diagnostic tool for the early detection of lung abnormalities. In contrast, it is a valuable tool for monitoring (day after day) the rapid progression of lung abnormalities in infected patients, particularly in intensive care units[33]. Despite its limited sensitivity, the appearance of a local or bilateral patchy shadow infiltrating a chest X-ray is the most typical radiological presentation[34].

Currently, CT plays a pivotal role and is the main technique for diagnosing and following patients with COVID-19[35]. The CT finding is more sensitive than the chest x-ray, particularly in the initial assessment[32,36]. CT findings may be present early, even before the onset of the symptoms[36]. Additionally, Li Y. and L. Xia's comparative study reflected the low misdiagnosed rate of CT scans and detected positivity earlier than RT-PCR[37]. The most common chest CT findings included ground-glass opacity, ill-defined boundaries, smooth or uneven interlobular septal thickening, an air bronchogram, a crazy-paving pattern, and thickening of the nearby pleura[38]. Due to numerous drawbacks, chest CT has some restrictions; for instance, radiation exposure, overuse of health care resources, hygiene, or inability to get a CT scan, as in critically ill patients, or clinically unstable, as in the case of intensive care unit (ICU) admission[39]. As a result, other methods are required to define and monitor patients rapidly.

Moreover, clinical pathologists have a significant role in monitoring inflammatory markers, including C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and white blood cells (WBCs). The most significant markers during SARS-COV-2 infection and highly associated with COVID-19 progression were lymphocytopenia, elevated CRP, and alternation in the ESR levels[40-42]. Data obtained from 452 patients with COVID-19 revealed that lymphocytopenia, high WBCs, a high neutrophil-lymphocyte ratio, and lower percentages of monocytes, eosinophils, and basophils were mainly observed in severe cases[43]. Similar findings were demonstrated by Henry *et al*[44] (2020) in their meta-analysis of 21 studies that included 3377 individuals who tested positive for COVID-19. They found that patients with severe and fatal diseases had more dramatic leukocytosis and lymphocytopenia and thrombocytopenia than mild to moderate diseased and survivor patients. The study by Mardani *et al*[41] (2022) attempted to explain the association between the inflammatory markers and COVID-19 progression and found that elevated CRP was correlated with the severity of COVID-19; furthermore, high ESR levels were observed in the severe cases only. Additionally, interleukin (IL)-7, IL-8, IL-9, IL-10, granulocyte-colony stimulating factor, granulocyte-macrophage colony-stimulating factor, tumor necrosis factor-alpha (TNF-α), and vascular endothelial growth factor A (VEGFA) were all found at high blood levels in COVID-19 patients[45].

In contrast, children show inconsistency and require further investigation. According to Del Valle *et al*[46] (2022) children with SARS-CoV-2-associated community-acquired pneumonia have low CRP levels. Additionally, a systematic review by Patel NA (2020) describes 2914 pediatric patients with COVID-19, the lab results for these children indicate stable WBC, lymphocyte count, and CRP levels[47]. Even though pneumonia causes an elevated CRP level, pneumonia with COVID-19 causes a drastic increase in CRP. This was revealed in a retrospective comparative study by analysis of the laboratory markers among children affected with pneumonia in the presence or absence of SARS-CoV-2 infection[48]. A meta-analysis study covers 20 eligible studies to identify the laboratory abnormalities among 1810 pediatric patients including Leukopenia, lymphopenia and elevated CRP[49]. Furthermore, the major conclusion of a retrospective cohort study by Graff *et al*[50] (2021), which included 454 patients, was that elevated CRP is a predictor of severe COVID-19 in children. All the previous studies show defects in the number of people involved in the studies. Hence, we recommend further investigation into many children.

Numerous investigations have demonstrated that liver damage occurred in SARS patients. This damage primarily took the form of mild to moderate elevations of alanine aminotransferase (ALT) or aspartate aminotransferase (AST) in the early stages of the illness. Some individuals' blood albumin

levels dropped as their bilirubin levels increased[51]. Compared to moderate cases, patients were more likely to have severe hepatic damage[52]. According to recent investigations into COVID-19, liver damage can occur in between 14.8% and 53% of cases, with aberrant ALT/AST values and slightly increased bilirubin levels serving as the significant indicators[53]. Severe cases reduced albumin (26.3-30.9 g/L)[54]. In recent research, including 1100 Chinese patients, Guan *et al*[34] found that 56% of patients with a severe COVID-19 infection and about 18% of patients with a non-severe COVID-19 disease had increased blood AST levels. Additionally, it was shown that patients with a non-severe COVID-19 illness accounted for 20% of patients with increased blood levels of ALT. In contrast, patients with severe COVID disease constituted 28% of patients. In COVID-19 fatality cases, liver damage occurred between 58.06% and 78% of the time[55]. A study showed that a patient with severe COVID-19 had blood ALT and AST values of 7590 and 1445 U/L, respectively[54].

RISK FACTORS

Intriguingly, lifestyle characteristics such as smoking, a high body mass index (BMI), male gender, postmenopausal status, and higher age in females were cited as the most significant risk factors for SARS-CoV-2 infection, regardless of comorbidities[56-58]. According to some studies, the age for an elevated risk is > 64 or > 65 years old. With six records, hypertension[59] and diabetes[60] are the most prevalent pre-existing comorbidities, followed by cardiovascular disease with three records. On rare occasions, associations were found between severity and TB, chronic renal illness, chronic obstructive pulmonary disease, or cerebrovascular disease. Significant effects on disease severity were reported for eight comorbidities that emerged because of COVID-19 infection[61]. Among them are organ failure, immune dysfunction, acute liver damage, hypoproteinemia, acute RDS, severe pneumonia, an uncontrolled inflammatory response, and hypercoagulable conditions[58,61].

Because their host defenses are compromised, patients with pre-existing liver diseases, such as cirrhosis of the liver and hepatocellular carcinoma (HCC), are more susceptible to infections and sepsis in general. Chronic liver diseases (CLDs) were present in 0.6% to 1.4% of hospitalized COVID-19 patients[62,63]. These individuals were more likely to experience severe illness (up to 60%) and increased death (up to 18%)[64]. Additionally, SARS-CoV-2 infection worsened the clinical prognosis and exacerbated liver damage in persons with CLDs resulting in decompensation in 20% of cirrhotic patients and worsening the clinical outcomes of people who were unstable[65].

The relationship between metabolically associated fatty liver disease (NAFLD) and COVID-19, among instances of chronic liver disorders and COVID-19, has received full attention. According to two investigations by Qian *et al*[66] and Ji *et al*[67], Patients with NAFLD have a longer viral shedding period and are more likely to have abnormal liver functions from the time of admission until discharge. Moreover, other investigations reported the same findings, with more significant mortality in patients with NAFLD, obesity, and those over 60 years old[68].

Additionally, the chance of rapid SARS-CoV-2 infection and developing COVID-19 complications appear with immunomodulatory and immunosuppressive drugs mainly used in autoimmune liver diseases. Therefore, patients with autoimmune hepatitis receiving immunosuppressive therapy should be viewed as having a high risk of developing severe COVID-19[69]. In contrast, the incidence of SARS-CoV-2 infection in patients with autoimmune hepatitis was like the general population, and the prevalence of severe COVID-19 was low[70]. Hence, we recommended further studies on patients with autoimmune hepatitis receiving immunosuppressive therapy.

Finally, according to preliminary findings on coinfection with SARS-CoV-2 and other viruses, it seems to cause severe progression, poor outcomes, or vial reactivation as in the case of the hepatitis C virus (HCV) and Hepatitis B virus (HBV) coinfection[71-73].

CLINICAL CHARACTERISTICS OF LIVER INJURY IN COVID-19

Recent research shows that the frequent symptoms of fever and cough coincide with the beginning of COVID-19 infection. Other clinical characteristics, such as diarrhea, nausea, vomiting, and lack of appetite, represent at least a digestive system symptom[34]. CoV infection has been linked to liver damage in SARS and Middle East respiratory disease patients[74]. In cases with COVID-19, abnormal liver function was observed, shown as isolated elevations in blood transaminase and lactate dehydrogenase (LDH) levels[75]. Alkaline phosphatase (ALP), LDH, ALT, AST, and prothrombin time levels gradually increased during the hospitalization of the first COVID-19 case in the United States[76]. According to a study from Jin Yin-tan Hospital, out of the 99 patients with COVID-19, 43 had ALT or AST levels above the normal range, 75 had elevated LDH levels, and one had a severe disruption in liver function[54]. With 3.75% of all cases in Jiangsu province being imported and cases outside Wuhan, liver damage was said to be less common in these patients[77]. In an analysis of liver function among patients outside intensive care units, males were more likely to experience liver impairment than females[78]. In pediatric instances, liver damage was discovered in 22% of kids, most often between 2

and 18 d after admission[79]. In Wuhan, liver injury is a common factor among patients who are admitted to the ICU and non-survivors hospitalized patients. This reflects the relationship between liver injury and the severity of COVID-19[80]. Fifty-two patients who required mechanical breathing or had at least 60% inspired oxygen been included in a study of critically ill individuals. Twenty-nine percent of patients with critical conditions had liver damage. Fifteen percent had acute renal disease, and fifteen percent had cardiac injury[81]. In a multicenter study involving 1099 patients and 552 hospitals, abnormal liver function was generally detected in critically ill participants, whereas jaundice was less frequently observed in COVID-19 patients. In harmony with the elevation of total bilirubin levels in 10% of patients, the percentage was increased in severe cases up to 20.5%[34]. Furthermore, a multicenter retrospective cohort study including 5771 patients in Hubei province suggested that upregulation in liver injury markers, particularly AST, is closely correlated with the probability of death during COVID-19[75]. Therefore, the dynamic patterns of liver injury markers and their putative risk variables may provide a significant explanation for the liver damage linked to COVID-19. Additionally, all studies indicated that liver injury parameters should be monitored during hospitalization.

POSSIBLE MECHANISMS OF COVID-19-ASSOCIATED LIVER INJURY

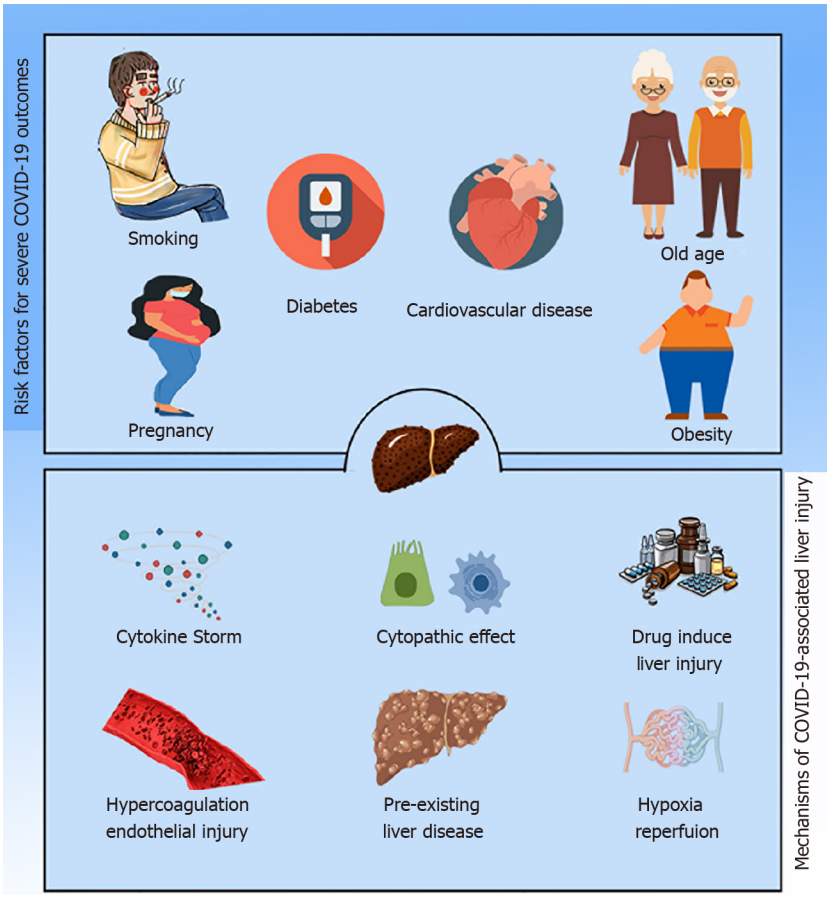
SARS-CoV-2 tropism and liver injury

ACE2 expression aroused the curiosity of researchers and scientists due to unusual ACE2 hepatic distribution and unexpected outcomes. Chai and colleagues assumed the hepatic abnormalities during COVID-19 were ascribed to cholangiocytes dysfunction, not due to hepatocytes damage (Figure 1). Their investigation using single-cell RNA-seq revealed that the primary target for SARS-CoV-2 in the liver was cholangiocytes. The ACE2 expression in hepatocytes is 20 times less than observed in cholangiocytes. Despite this, clinical data from COVID-19 patients showed rising ALT, AST, and LDH levels, while ALP and gamma-glutamyl transferase, which describe bile duct injury, did not significantly increase[82]. At the same time, histological and immunohistochemistry assessments of Kupffer cells and T and B lymphocytes did not express ACE2[83], even though COVID-19-infected patients' livers frequently showed Kupffer cell activation and proliferation[84,85]. Additionally, systemic inflammation typically results in Kupffer cell activation and proliferation[84]. Although Kupffer cells do not express, ACE2 may have a crucial role in the propagation of inflammation that results in SARS-CoV-2-mediated liver damage. It is noteworthy that prediction of SARS-CoV-2 consecutive signaling, and outcome is challenging because the expression of ACE2 level is regulated by many factors and conditions, for example, liver fibrosis, liver cirrhosis, hypertension, diabetes, chronic pulmonary diseases, hypoxia, old age, and smoking, which represent factors for COVID-19[17,86,87].

SARS-CoV-2 uses ACE2 receptors to invade host cells and utilizes other molecules to facilitate infection, such as furin, transmembrane serine protease 11A (TMPRSS11a), and neuropilin-1[88,89].

Neuropilin-1 is embedded in the liver, causing physiological and pathological conditions. Activation of the neuropilin-1 cascade triggers angiogenesis process *via* controlling cell proliferation, cell survival, and cell migration[90]. Regardless of the cause of hepatic injury and conditions resulting from a viral infection, the elevation of neuropilin-1 is the defense mechanism. Consequently, neuropilin-1 may influence liver damage induced by SARS-CoV-2[89]. Neuropilin-1 has been reported to be found and expressed in liver sinusoidal endothelial cells and hepatic stellate cells[91]. Meanwhile, hepatic stellate cells' activation is postulated to be the primary cause of liver disease and fibrosis[92,93]. In different conditions, the hepatic stellate increases proinflammatory and profibrotic cytokines[94]. One of those cytokines is IL-6, produced when SARS-CoV-2 activates the immune system in COVID-19 patients and is associated with altered liver enzyme levels[95]. Therefore, propagation of neuropilin-1 expression with activation of hepatic stellate cells promotes signaling transcription and stimulates the release of growth factors such as transforming growth factor -β and VEGF, elucidating their role in the progression of liver damage during SARA-CoV-2 infection[96].

All the data mentioned above are consistent with a detailed histological examination clarifying the possible mechanisms of hepatic injury. Wang *et al*[84] uncovered the presence of intact SARS-CoV-2 viral particles in the cytoplasm of hepatocyte samples obtained from 156 dead COVID-19 patients. Further observations revealed conspicuous mitochondrial swelling, endoplasmic reticulum dilatation, glycogen granule decrease, fibrin deposition, granulomas, massive central necrosis, and apoptosis. Another study by Fiel *et al*[97], using in situ hybridization and electron microscopy, reported that SARS-CoV-2 directly invades liver cells and induces histological changes such as apoptosis, especially in cholangiocytes, abundant mitoses, mixed inflammatory infiltrates in portal tracts, endothelins, and severe bile duct damage. In a case study by Melquist *et al*[98], the direct SARS-CoV-2 cytopathic effect caused a rapid progression of acute hepatitis to fulminant liver failure with a mild increase in transaminase levels without developing respiratory symptoms. Data from the international study involving 130 centers in 29 countries revealed that the stage of liver disease is closely correlated with COVID-19 mortality. The highest rates of hepatic decompensation and mortality were observed in patients with advanced liver cirrhosis and those with alcoholic liver disease[99].



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Figure 1 Risk factors and possible mechanisms of COVID-19-associated liver injury. COVID-19: Coronavirus disease 2019.

Cytokine storm and liver injury

SARS-CoV-2 induces immune dysregulation associated with the unspecified release of proinflammatory cytokines and coagulation enzymes. The massive release of cytokines is known as a cytokine storm or cytokine release syndrome and is characterized by the magnitude of the release of interferons, TNFs, ILs, and chemokines[100]. Hence, uncontrolled systemic proinflammatory cytokine release represents unfavorable clinicopathological conditions in COVID-19 patients, for instance, progressive liver damage and liver failure.

IL-6 is the most significant cytokine in liver hepatocytes and is a crucial inducer of the acute phase response and infection defense[101]. IL-6 stimulates hepatocytes during the initial phase of inflammation to upregulate CRP, fibrinogen, haptoglobin, alpha-antitrypsin, and serum amyloid-A which induce acute inflammatory phase[101]. Additionally, prolonged inflammation stimulates IL-6, targeting monocyte chemotaxis toward tissue-destructive injury[102]. Furthermore, IL-6 induces multiple effects during the storm *via* the activation of different transduction signaling pathways, *e.g.*, nuclear factor *kappa*-light-chain-enhancer of activated B cells (NF-κB), janus kinase (JAK)/ signal transducer and activator of transcription (STAT), and the Akt/Phosphatidylinositol-3-kinase pathway[71,103].

Similarly, attention must be paid to the crucial roles of the ACE/Ang II/ angiotensin II receptor type 1 pathways. Ang II can directly activate the NF-κB pathway, increasing the secretion of IL-6, IL-1β, TNF-α, and IL-10[104]. Moreover, Ang II has been reported to induce mitogen-activated protein kinases activation, which in turn induce pro-inflammatory cytokines' release[105].

A case series study by Li *et al*[106], revealed elevated serum transaminase levels attributed to systemic inflammation, cytokine storm syndrome, and hepatocyte damage. Darif *et al*[95] reported that hepatic injury in patients with COVID-19 was attributed to systemic inflammation. Therefore, significant elevations in CRP, TNFα, and IL-6, concomitant with a significant elevation in aminotransaminase, describe hepatic injury associated with SARS-CoV-2 infection[107,108]. All these data confirm the relationship between inflammation during COVID-19 and hepatic injury.

Hypoxia and liver injury

One of the most common complications of COVID-19 is acute respiratory distress syndrome requiring a high level of management[81,109,110]. COVID-19 is associated with impaired respiration, an insult to blood flow, and hypotension, which are clues to hypoxic hepatitis, and might exacerbate liver damage

or even lead to liver failure[11,106]. Ischemia induces profoundly detrimental cellular effects and results in metabolic abnormalities, for example, disturbances in lipid metabolism as well as lack of oxygen supply initiate hepatocellular death[112]. Furthermore, rapid recovery of blood flow with reoxygenation of hepatocytes results in metabolic abnormalities, the generation of reactive oxygen species, an inflammatory response, and cellular death[113]. Hence, hepatic ischemia deteriorates hepatic status *via* destructive cellular reactions concomitant with immune stimulation[112-114]. Hypoxia has been determined as the primary pathway to regulating ACE2 expression in hepatic cells[115]. These phenomena rapidly progress with a conspicuous elevation of transaminase levels, accompanied by LDH elevation[116]. A retrospective study by Huang *et al*[117] revealed that hypoxic hepatitis is apparent in intensive care units and is often associated with a drastic elevation in ALT levels, multiorgan damage, and high mortality risk. Additionally, patients with COVID-19 and hypoxic hepatitis are sometimes comorbid with respiratory failure, septic shock, or heart failure[53,80,118]. All these findings suggest an association between hepatic ischemia/hypoxia-reperfusion injury and liver injury during the SARS-CoV-2 infection.

Endothelial cells and liver injury

SARS-CoV-2 induces hypercoagulation, with the incidence of pulmonary embolism associated with complications aggravating heart failure and liver congestion[119]. Hypercoagulation and clotting disorders might occur through direct infection of platelets or a cytokine storm[120]. As mentioned above, patients with COVID-19 reported a change in platelet count and prothrombin time with an elevation in D-dimer and fibrinogen concentrations[80,121-123]. A multicenter, retrospective cohort study found that patients who died from COVID-19 were more likely to have severe hematological (lymphopenia, ferritin, and elevated D-dimer) and cardiogenic factors (troponin and lactate dehydrogenase), providing support for this hypothesis[80]. Goshua *et al*[123] reported that patients with COVID-19 showed a disturbance in epitheliopathy and platelet activation markers, particularly von Willebrand factor (vWF) antigen, P-selectin, and soluble thrombomodulin, anticipating a poor outcome or death. Furthermore, a case report study by Antunes de Brito *et al*[124] observed hepatic artery thrombosis in a patient with COVID-19 who experienced acute abdominal pain in harmony with elevations in protein C and D-dimer. Histological examination implied a severe disruption of the intrahepatic blood vessel network secondary to systemic changes induced by the virus that might also affect the cardiovascular system, coagulation cascade, and endothelial layer of blood vessels[125]. Additionally, a series of pathological examinations of liver autopsies obtained from deceased COVID-19 patients elucidated platelet aggregation in some portal veins as well as hepatic sinusoidal injury due to platelet-fibrin microthrombi[126]. However, ischemic-type damage in the liver has been observed in some cases [126]. Massive data indicate a relationship between hypercoagulation and liver injury in COVID-19 patients.

SARS-CoV-2 infection induces a pathological thromboinflammation response, including platelet hyperreactivity, hypercoagulability, and hypofibrinolysis[127]. SARS-CoV-2 binds to ACE2 receptors on the surface of endothelial cells and subsequently induces endothelial injury[127]. Additionally, SARS-CoV-2 invades megakaryocytes and platelets[128]. Endothelial cell activation and injury were confirmed by elevation of several blood hemostatic factors including vWF, thrombomodulin, and factor VIII[122, 123]. Collectively, they trigger a platelet plug activation[129,130]. A procoagulant molecule and platelet tissue factor, produced by hepatocytes and endothelial cells, attach, and activate factor VII, a procoagulant molecule that circulates in the blood. Activated factor VII activates factor X, which subsequently resulted in thrombin formation. Thrombin promotes a series of coagulation processes to produce fibrin which build a substantial fibrin mesh, in addition to platelet activation and aggregation [131,132]. Furthermore, Ang II increases plasminogen activator inhibitor-1 (PAI-1) expression in endothelial cells, which inhibits fibrinolysis and induces a hypercoagulable state[133].

Furthermore, hypoxia promotes coagulation through multiple pathways, such as hypercoagulation and inflammation. Hypoxia attenuates endothelial cells' anticoagulant function by suppressing thrombomodulin with increased PAI-1 upregulation. It promotes NF-κB and toll-like receptor 4 signaling pathways in macrophages and neutrophils, stimulating the release of IL-6 and TNFα[134-136].

Excessive inflammatory cytokines, particularly IL-6, facilitate SARS-CoV-2 and mediate coagulopathy[137]. IL-6 stimulates platelet formation and megakaryocytopoiesis generation, which could generate a hypercoagulability state[138]. A retrospective study by McConnell *et al*[139] revealed that the IL-6/JAK/STAT pathway is responsible for coagulopathy and hepatic epitheliopathy associated with COVID-19 and could be the potential mechanism of liver injury in these patients.

Drug-induced liver injury

Several medications can induce liver dysfunction and hepatocellular damage. Some are used as over-the-counter medications, for example, paracetamol, and others are used with precautions such as antibiotics, including azithromycin[140]. Although drug-induced liver damage is rare, it can immediately result in acute liver failure and require a liver transplant[141]. Drug metabolism is a possible cause of drug-induced liver injury (DILI) development by generating chemically reactive drug metabolites. The failure to metabolize reactive drugs can result in mitochondrial damage and oxidative stress, activating different signaling pathways[142]. Furthermore, reactive metabolites can act as haptens

and create neoantigens, which, when presented on human leukocyte antigen (HLA) molecules or attached to HLA molecules, can activate T cells, and trigger an adaptive immune response[143].

Several antiviral medications, supportive care, and trials of complementary therapies are among the therapeutic options being investigated against SARS-CoV-2. Hepatotoxicity from nucleoside analogs and protease inhibitors, which are used to manage COVID-19, can occur because the liver is involved in the metabolism of many medications. In a case study from Wuhan, after receiving lopinavir and ritonavir, the patient developed liver damage[143]. A recent randomized controlled study compared the elevation of AST, ALT, and total bilirubin in COVID-19 patients associated with lopinavir and ritonavir [144]. In a retrospective analysis of COVID-19, Fan *et al*[145] found that significantly elevated liver enzymes and liver abnormalities were in harmony with receiving combination therapy. In this study, 47.3% of the released patients had increased liver function tests (LFTs) at baseline, and 23.7% experienced abnormalities during hospitalization, which might be due to treatments or the disease.

It was discovered that many COVID-19 patients had previously used antipyretics and analgesics, most frequently paracetamol, whose overdose is recognized as a cause of liver injury with a significant elevation of serum aminotransferases[146]. Additionally, hepatic injury worsens in critical illnesses and patients with preexisting CLDs[147]. Therefore, healthcare providers should be aware of over-the-counter medications used to control common COVID-19 symptoms such as fever and pain. Physicians play a role in monitoring abnormalities in LFTs as they can indicate unknown drug hepatotoxicity.

Hydroxychloroquine is one of the drugs suggested for COVID-19 therapy regimens, an anti-malarial medicine that relies on scant data in limited clinical settings[148]. Based on clinical data, hydroxychloroquine hepatotoxicity during COVID-19 is rare[149]. A few incidences of significant increases in aminotransferases brought on by hydroxychloroquine have never been documented[150]. Therefore, patients with liver disorders should use hydroxychloroquine cautiously since it can accumulate[151].

Azithromycin is an antibacterial drug belongs to macrolide antibiotic. It was used to treat bacterial infections before treating COVID-19 alone or in combination with hydroxychloroquine[152]. Hence, the hepatic injury should be considered due to the high use of these medications. Liver damage may rarely occur within the first two to three weeks of starting azithromycin. Most patients fully recover from it, and it is predominately a hepatocellular pattern[153].

Remdesivir belongs to an adenosine analog with antiviral action[154]. A multicenter, randomized, double-blind, placebo-controlled trial by Wang *et al*[155] revealed that 10% of the remdesivir group had high blood bilirubin and 5% had increased aminotransferases. Additionally, Remdesivir was used to treat COVID-19 in a case series (n = 53), and 23% of the patients experienced elevated liver enzyme levels that required early treatment termination[156]. However, clinical data implied that the relation between remdesivir and hepatic injury during COVID-19 treatment needs more explanation[154].

To conclude, medications that reduce inflammation and preserve the liver should be given to individuals who are expected to experience liver damage, regardless of the drug, dosage, or dose[140].

All studies regarding possible mechanisms of COVID-19-associated liver injury were summarized in Table 1.

COVID-19 COMORBIDITY WITH DIFFERENT HEPATIC ILLNESSES

COVID-19 and viral hepatitis

HBV is a double-stranded DNA virus, a member of the *Hepadnaviridae* family. In contrast, HCV is a single-stranded RNA virus belonging to the Flaviviridae family[100]. Recently, several studies have indicated that the coinfection of COVID-19 and HCV is a predictor of acute-on-chronic liver failure and a high potential for ICU admission. A cohort study indicated that HCV patients with SARS-CoV-2 coinfection were more likely to be hospitalized. However, the mortality rate did not change[157].

In a retrospective cohort study that included 242 patients with hepatitis C cirrhosis, 46 patients were coinfected with SARS-CoV-2 and HCV and had high levels of ferritin, creatinine, blood urea nitrogen, prothrombin time, and HCV viral load, anticipating the development of acute-on-chronic liver failure and the potential for ICU admission[158]. An observational study by Toma *et al*[159] among patients with SARS-CoV-2, active HCV, and cure HCV in a control group showed the highest serum concentrations of ALT, AST, CRP, and ferritin. Moreover, serum and fecal calprotectin were detected in a patient with SARS-CoV-2 infection. In a serological study by León *et al*[160], patients with both HCV coinfection demonstrated a considerable elevation in IL-6 and IL-17, with lower TNF-α levels when compared with patients infected with HCV or SARS-CoV-2.

On the other hand, a nationwide population-based study has reported that patients infected with HBV were predisposed to have severe symptoms of SARS-CoV-2, a high probability of ICU admission, and more organ failures than patients without HBV infections, especially in older patients[161].

In addition, severe monocytopenia, lymphopenia, hypoalbuminemia, and lipid metabolism deficiency were observed in the liver of coinfected with SARS-CoV-2 and HBV[162,163]. Besides the elevation of liver impairment markers, including ALT, AST, ALP, and total bilirubin, several novel risk factors have been identify ed, including elevated LDH, D-dimer, decreased albumin, and albumin/globulin ratio[164]. However, other studies have found that HBV is not related to the poor outcomes of

Table 1 Explore the main causes of liver injuries during COVID-19

Cause of liver injury	The main finding of the study	Ref.
	SARS-CoV-2 directly invades the liver and displays hepatic impairment characterized by liver enzyme abnormalities	Wang *et al*[84], 2020
SARS-CoV-2 tropism	Intrahepatic SARS-CoV-2 contributes to liver inflammation, endothelium, and bile duct damage	Fiel *et al*[97], 2021
	SARS-CoV-2 cytopathic effect involved in the rapid progression of acute liver injury to acute liver failure	Melquist *et al*[98], 2020
Cytokine storm	Elevation of liver enzymes in COVID-19 is mainly related to immune dysregulation caused by cytokine storm and hepatic damage	Li *et al*[106], 2020
	Systemic inflammation is the fuel for hepatic injury in COVID-19 patients	Effenberger *et al*[244], 2021
Hypoxic liver injury	Hypoxic hepatitis is not a rare condition in COVID-19 patients admitted to the intensive care unit and is dramatically associated with elevated liver enzymes	Huang *et al*[117], 2020
	Hepatic artery thrombosis is highly associated with hepatic injury and abdominal pain during COVID-19	Antunes de Brito *et al*[124],2021
	SARS-CoV-2 induces severe disruption of the intrahepatic blood vessel and also affects the endothelial layer of blood vessels	Sonzogni *et al*[125], 2020
Endothelial cells and liver injury	Hepatic injury is attributed to platelet-fibrin microthrombi in the hepatic sinusoids along with some portal vein platelet aggregates	Rapkiewicz *et al*[126], 2020
	SARS-CoV-2 activates IL-6/JAK/STAT pathway consequently, stimulating coagulopathy and hepatic epitheliopathy	McConnell *et al*[139], 2021

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; COVID-19: Coronavirus disease 2019; IL-6: Interleukin-6; JAK: Janus kinases; STAT: Signal transducer and activator of transcription proteins.

COVID-19[165]. Furthermore, the reactivation of HBV may occur due to the COVID-19 vaccine, as observed in some cases[166-168].

COVID-19 and viral hepatitis during pregnancy and its impacts on neonates

Acute HBV infection during pregnancy is not a risk factor for fetal death or teratogenicity. However, many complications in HBV-infected pregnant women may be associated with an increased risk of gestational diabetes, postpartum hemorrhage, premature birth, and low birth weight[169]. Furthermore, in a prospective cohort study, Rajan *et al*[170] indicated that pregnant women with both HBV and SARS-CoV-2 coinfection had a high proportion of preterm deliveries and a low mean birth weight. In rare cases, the coinfection of both viruses has led to intrahepatic cholestasis in pregnancy and acute fatty liver disease of pregnancy (AFLP)[171]. Nevertheless, there is some indication that HBV and COVID-19 coinfection does not lead to worse results[170,172]. On the other hand, some studies have provided evidence that treatment regimens including antivirals, hepatoprotective, and low-dose dexamethasone drugs might be recommended in cases of pregnant women with HBV and COVID-19 coinfection, besides coagulation function monitoring as part of the management process[171,173].

Similarly, pregnant women with HCV infection are more likely to have infants born prematurely, stillborn infants, newborns with low birth weight, or infants with birth abnormalities[174,175]. Furthermore, from an epidemiological point of view, the worldwide hepatitis elimination program has been affected due to COVID-19 spreading, and this may require new policies and strategies for hepatitis elimination[176-178].

Ahmed *et al*[179] reported a case-report study in which a 26-year-old Asian female pregnant patient was affected by a sudden onset of severe preeclampsia complicated by AFLP and acute kidney injury (AKI) following SARS-CoV-2 infection. Besides, the comorbidities of SARS-CoV-2 and preeclampsia in pregnancy can lead to AFLP and AKI. This comorbidity can cause calcifications of the bowel and gallbladder of the fetus[180,181], besides a liver parenchymal disease associated with liver rupture[182], liver coagulation, liver impairment, and preterm delivery[183]. Furthermore, a pregnant woman with SARS-CoV-2 infection at 28 wk with a low-lying placenta was complicated by obstetric cholestasis and several episodes of minor antepartum hemorrhage[184]. Moreover, placental insufficiency and subsequent fetal hypoxia may occur[185].

COVID-19 and pregnancy: Several mechanisms for complications

Recently, pregnant patients who were coinfected with SARS-CoV-2 showed a higher risk of developing complications than those who were not pregnant. Studies have shown that pregnant women with SARS-CoV-2 infection increased the probability of developing preeclampsia compared to individuals who did not have SARS-CoV-2 infection during pregnancy[186]. Nevertheless, symptomatic patients were more

likely to have preeclampsia than asymptomatic ones[186,187].

On the other hand, several hypotheses may illustrate the high rate of preeclampsia associated with SARS-CoV-2 infection. A direct cytopathic effect with dysregulation of the RAAS system induces a change in the placenta's function[188-191] because it controls the proliferation of trophoblasts, angiogenesis, and placental blood supply. Thus, the interaction between SARS-CoV-2 and ACE2 receptors described in RAS system down-regulation and reduction of vasodilatory angiotensin 1 to 7 results in continuous vasoconstriction and pro-inflammatory effects of angiotensin II, which finally lead to a pathophysiological mechanism of preeclampsia[192-196]. A study conducted by Verma *et al*[197] suggested that the infected placenta had a reduction in ACE2 receptor expression, proangiogenic factors, and an increase in the production of soluble FMS-like tyrosine kinase-1 (sFlt-1), which are biomarkers for preeclampsia. An in-silico study by Seethy *et al*[198] concluded that interactions between SARS-CoV-2 and the placenta are regulated through trophoblast invasion, migration, proliferation, and differentiation processes by the milk fat globule-EGF factor 8 protein, plasminogen activator, and protease-activated receptor 2 proteins.

In parallel, pregnant women might be able to develop a pre-eclampsia-like syndrome characterized by proteinuria, hypertension, thrombocytopenia, the elevation of liver enzymes, an abnormal uterine artery pulsatility index, and increased sFlt-1/placental growth factor[199], besides preeclampsia, coagulopathy, and the HELLP (hemolysis, elevated liver enzymes, low platelet count)[200].

COVID-19 and liver fibrosis/cirrhosis

Recently, it has been hypothesized that patients with a hepatic illness have a higher mortality rate after SARS-CoV-2 infection. Non-invasive indices, including the Fibrosis-4 index (FIB-4), the NAFLD fibrosis score, and the AST to platelet ratio index, have been developed to determine the severity of fibrosis, which plays a crucial role in assessing liver fibrosis[201]. In a multicenter observational study, Kim *et al*[202] reported that patients with diabetes mellitus (DM) showed a higher FIB-4 index, serious complications such as severe respiratory failure, venous thromboembolism, hepatic injury, and a high mortality rate compared to patients without DM. Meanwhile, the FIB-4 index might be used to assess the risk of progression to hepatic illness in middle-aged patients with COVID-19[203]. An association was observed between liver fibrosis scores and poor outcomes, and these findings were consistent with previous research that found worse outcomes in COVID-19 individuals with pre-existing chronic liver disorders, including a high proportion of ICU admission and the need for mechanical ventilation[204, 205]. An explanation for liver injury could be the presence of high levels of lymphocytes and natural killer cells inside the hepatic tissue[206].

On the other hand, An *et al*[207] conducted a STROBE observational study and reported that patients with liver cirrhosis and COVID-19 were frequently admitted to the hospital more than those with liver cirrhosis only. Unlikely, in the same study, cirrhotic patients who lacked COVID-19 experienced more severe liver cirrhosis-related consequences and needed immediate treatment. In a multicenter cohort study, Bajaj *et al*[208] illustrated that those with cirrhosis alone or with COVID-19 had equal death rates, while patients with COVID-19 alone had a greater mortality rate.

COVID-19 and liver fibrosis/cirrhosis during pregnancy

As discussed above, having an infection makes pregnant women more susceptible to developing more severe symptoms. Biomarkers such as ALT, AST, ALP, elevated D-dimer levels, fibrin degradation, and prolonged prothrombin time lead to liver injury, liver fibrosis, and liver cirrhosis; hence, increasing the possibility of preeclampsia with HELLP syndrome[179].

COVID-19 and HCC

HCC is the third most important cause of cancer-related mortality and the sixth most frequent cancer in the world. SARS-CoV-2 virus infection has recently been considered a risk factor for cancer patients because SARS-CoV-2 might aggravate liver damage in HCC patients[209]. Furthermore, a US multicenter study by Kim *et al*[210] reported that having HCC indicates a greater mortality rate in individuals with HCC infected by SARS-CoV-2 than COVID-19 alone, especially in patients with obesity, DM, hypertension, hyperlipidemia, older patients (≥ 65 years), and Hispanic ethnicity. Also, in China, patients with HCC and COVID-19 were shown to be more susceptible to a higher risk of death and admission to the ICU[211]. In parallel, Leo *et al*[212] retrospectively analyzed 119 patients with HCC and COVID-19 infection. They found that about one-third of patients required hospital admission. Two-thirds had an elevation of transaminases, particularly ALP, which was independently linked to a high mortality rate, higher CRP levels, and more severe respiratory failure upon admission to the hospital.

Liver transplantation and COVID-19

According to the American Society of Transplantation, there has yet to be an agreement on the ideal timing of liver transplantation (LT) in patients infected with SARS-CoV-2. However, it is recommended that before transplantation, recipients should have a negative SARS-CoV-2 test[213]. Nevertheless, Martinez-Reviejo *et al*[214] determined that, regardless of symptoms at the time of infection, using LT from SARS-CoV-2 positive donors appears to be a safe technique with a low risk of transmission.

Furthermore, a multicenter network study by Mansoor *et al*[215] found that LT patients with COVID-19 had a substantially larger possibility of hospitalization but not mortality, thrombosis, or ICU admission when compared to those without LT and COVID-19. In contrast, a case-control study by Shafiq *et al*[216] stated that regarding death and hospitalization rates, there was no significant difference between the case and control groups in liver enzyme ratios, and both had a normalized value at the time of discharge. In addition, the only difference in the patient's pathological characteristics is the type of liver graft, alkaline phosphatase levels, and lymphovascular invasion[217]. A case-report study indicated that some LT could be successful in active SARS-CoV-2 patients without developing post-operative COVID-19 symptoms[213]. Furthermore, an Italian multicenter series by Romagnoli *et al*[218] found that liver transplantation from COVID-19-positive donors to informed recipients with SARS-CoV-2 immunity might help increase the safety of the donor pool. Rela *et al*[219] reported a successful LT in patients with severe liver failure due to cholestasis with good graft function and recovering function in the native liver remnant.

Collectively, the effect of comorbid hepatic disorders with SARS-CoV-2 infection was summarized in Table 2.

VACCINES USED FOR COVID-19 PREVENTION

Recombinant DNA, mRNA, and adenovirus vector-based technologies were the three main methods of vaccine development that demonstrated immediate success. All have been shown to help prevent infections, especially in severe diseases, because breakthrough infections are typically asymptomatic or mild-to-moderate. BNT162b2 (Pfizer-BioNTech) and mRNA-1273 (Moderna) were emergently approved in the United States as the first mRNA vaccines[220]. Following that, an Emergency Use Authorization (EUA) license was granted for the two most effective adenovirus-based vaccinations in the United States (Ad26.COV2.S) (Janssen-Johnson & Johnson) and Europe (ChAdOx1.nCoV-19; Oxford-Astra Zeneca). Adenovirus-vectored vaccines have demonstrated effectiveness in China (Ad5-vectored COVID-19 vaccine) and countries that produce traditional, inactivated viral vaccines. The most frequently used COVID-19 vaccinations are intramuscular injections, and a first dose is recommended to be followed by a second dose within three to four weeks. Currently, a booster dose is recommended administrated after six months of the initial immunization. Individuals 18 years of age and older may get a booster dose of the Johnson & Johnson COVID-19 vaccination 2 mo following the initial single dose[221,222].

Pfizer-BioNTech vaccine (BNT 162b2)

An intramuscular mRNA vaccine called BNT 162b2 is administered in two doses (30 μg *per* dose) at 21-d intervals. The vaccine is accessible in multidose vials and must be refrigerated at a temperature between 60 °C and 80 °C[223], which might present a logistical challenge in developing nations. According to phase I/II/III, randomized, placebo-controlled trials published in December 2020, the Food and Drug Administration (FDA) approved it for emergency use[224]. In the study, 43448 volunteers were randomly assigned in a 1:1 ratio to the vaccination arm and the placebo arm. Compared to the placebo, the vaccination showed a 95% efficiency in preventing COVID-19, and this efficacy was maintained for subgroups based on age, sex, BMI, ethnicity, and comorbidities. Local site responses were the most prevalent adverse effects. Young patients were more likely to experience systemic symptoms such as fever, joint discomfort, and chills, which increased following the second dosage[225]. Just three individuals with moderate or severe liver disease were included in the trials, with 214 participants having mild liver disease. The virological status and disease severity of patients with HBV and HCV infections were included; however, it was unknown how severe their conditions were. Furthermore, immunosuppressive drug users were excluded. Hence, more information is required concerning people with liver illnesses[226,227].

Moderna vaccine (mRNA-1273)

The mRNA-1273 is another mRNA vaccination given in two doses of 100 μg each, separated by 28 d. Based on phase III randomized placebo-controlled trial published in December 2020, in which 30420 participants were randomly allocated to the immunization and placebo groups in a 1:1 ratio, the FDA approved the vaccine. The effectiveness of the vaccination in preventing COVID-19 was 94.1%. Only the placebo group experienced severe COVID-19, resulting in one participant's death. Serious, unanticipated adverse reactions to vaccinations were more frequent in the vaccine group, but none were fatal or forced to be completed until the research's end. After the second dose and in younger people, the unwanted local and systemic responses were more prevalent[228,229]. Although the liver condition was not specified, the study included 196 individuals with liver disease (divided equally between the vaccination and placebo groups). Participants in the experiment who were on systemic immunosuppressive medication were not allowed. For individuals with hepatic illness, no independent efficacy and safety data were available[228].

Table 2 Summarizing the effect of comorbid hepatic disorders with SARS-CoV-2 infection

Hepatic disorders	Main finding	Ref.
	SARS-CoV-2 comorbidity with HCV shows a high percentage of ferritin, white blood cell count, prothrombin time, lymphocyte count, and hypoglycemia	Cerbu *et al* [159], 2022
	SARS-CoV-2 and HCV coinfection reported higher levels of IL-6 and IL-17, and TNF-α when compared with HCV and COVID-19 alone	León *et al*[161], 2022
HCV	The Serum levels of ALT, AST, CRP and ferritin, and calprotectin were significantly elevated in patients with COVID-19 infection than in patients with active HCV and patients with cured HCV infection	Toma *et al* [160], 2022
	HCV patients with SARS-CoV-2 infection are more likely to be hospitalized with a high possibility of liver fibrosis and mortality	Butt *et al*[158], 2021
	Individuals with HCV and SARS-CoV-2 co-infection are more vulnerable to developing liver cirrhosis	Afifyet al[245], 2021
	Patients with a history of HBV are anticipated to have a worse outcome with a high probability of ICU admission, and more organ failures	Choe *et al*[162], 2022
	S SARS-CoV-2 and chronic HBV showed severe monocytopenia, lymphopenia, thrombocytopenia, hypoalbuminemia, and lipid metabolism deficiency in the liver	Zou *et al*[164], 2021
HBV	Patients with HBV and SARS-CoV-2 coinfection died from severe liver disease and haptic sclerosis	Chen *et al* [163], 2020
	Patients with HBV who have COVID-19 were more likely to develop devastating illnesses and/or death. Additionally, the elevation of LDH, and D-dimer, with decreased albumin, and albumin/globulin ratio is helpful for early clinical surveillance	Wang *et al* [165], 2022
	Patients with DM with advanced liver fibrosis infected by SARS-CoV-2 are assumed to have a 10-time risk of mortality when compared with patients without comorbidities	Kim *et al*[203], 2021
	The high proportion of ICU admission, and the need for mechanical ventilation	Hassnine *et al* [206], 2022
Liver cirrhosis	Patients with liver cirrhosis and COVID-19 were admitted to the hospital than liver cirrhosis alone	An *et al*[208], 2021
	Those with cirrhosis alone or cirrhosis with COVID-19 had equal death rates, while patients with COVID-19 alone had a greater mortality rate	Bajaj *et al*[209], 2021
	HCC predicts a greater mortality rate in individuals with HCC infected by SARS-CoV-2 than COVID-19 alone, especially in patients with obesity, diabetes mellitus, hypertension, and hyperlipidemia, older patients ≥65 years, and Hispanic ethnicity	Kim *et al*[211], 2021
HCC	HCC and COVID-19 were shown to be more susceptible to have a higher risk of death and admitted to the ICU	Liang *et al* [212], 2020
	Patients with HCC-COVID-19 coinfection found that about one-third of patients need hospital admission, and two-thirds of patients have an elevation of transaminases. Alkaline phosphatase which independently linked to a high mortality rate, higher C reactive protein levels, and more severe respiratory failure upon admission to the hospital	Leo *et al*[213], 2022
	LT patients with COVID-19 had a considerably increased risk of hospitalization but not a significantly higher risk of mortality, thrombosis, or need for ICU admission	Mansoor *et al* [216], 2021
	High alkaline phosphatase levels, and lymphovascular invasion	Shafiq *et al* [217], 2022
LT	LT cases could be successful in active SARS-CoV-2 patients without developing post-operative COVID-19 symptoms	Mouch *et al* [214], 2022
	Found that liver transplantation from COVID-19-positive donors to informed recipients who have SARS-CoV-2 immunity may help to increase the donor pool safely	Romagnoli *et al* [219], 2021
	Successful LT In patients with severe liver failure due to cholestasis with a good graft function and recovering function in the native liver remnant	Rela *et al*[220], 2022

HCV: Hepatitis C virus; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; COVID-19: Coronavirus disease 2019; IL-6: Interleukin-6; IL-17: Interleukin-17; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CRP: C-reactive protein; HBV: Hepatitis B virus; ICU: Intensive care unit; HCC: Hepatocellular carcinoma; LT: Liver transplantation.

ChAdOx1nCoV-19 vaccine (AZD1222)

ChAdOx1 nCoV-19 vaccine (AZD1222) was created by the University of Oxford, which uses a replication-deficient chimpanzee adenovirus as a vector containing the gene encoding for the SARS-CoV-2 spike glycoprotein. Storage conditions may be kept between 2 and 8 °C and are less strict than mRNA vaccines. AstraZeneca and Serum Institute of India produce it (SII). In December 2020, the UK

granted emergency use authorization for the vaccine produced by AstraZeneca. The vaccine, produced by SII under the brand name COVISHIELDTM®, was approved for use in India by the Drug Controller General of India[229]. Two intramuscular vaccine doses, each containing 0.5 mL, were given over a 4–6 wk interval. In patients who got a single dose, antibody responses peaked on day 28, and in individuals who received a booster dose four weeks later, they peaked on day 56[228,229]. A pooled intermediate analysis of four randomized controlled trials by Voysey *et al*[230] conducted in Brazil, South Africa, and the UK, which included 23848 people, was used to support the authorization. Of these, 11636 patients were included in the interim study. The experiment showed total vaccination effectiveness of 70.1%. After 21 d following immunization, 10 COVID cases were recorded; all were in the control group and included two cases of severe COVID and one case of death. In addition, only three of the 175 cases with adverse effects might have been caused by vaccination. Individuals with hepatic disorders were mostly excluded from the 4 studies described above. Patients with severe liver diseases were not included in the trials in the UK and Brazil, although the severity standards were unclear. Furthermore, individuals using immunosuppressive drugs and those with alcohol dependence were excluded. Abnormal LFTs, Australian antigen-positive status, CLDs, and alcohol misuse were listed as exclusion criteria in a South African study. Only two individuals (one from each vaccination and control group) had abnormal liver function[231].

Janssen vaccine/Ad26.COV2.S

This full-length SARS-CoV-2 S protein-containing non-replicating human adenovirus type 26 triggers an immune response to the SARS-CoV-2 infection. The SARS-CoV-2 virus is prevented from invading type 2 alveolar cells in the lungs by an antibody directed against the S protein, lessening the severity and morbidity of the infection[232]. Adjuvant properties, scalability, and broad tissue tropism are benefits of adenoviral vectors[233,234]. Since these labs need biosafety level 2 certification, vaccine production will likely go more slowly during this pandemic. Additionally, a person with immunity to viral vectors would reduce the vaccine's efficacy. Employing the chimpanzee adenovirus (ChAdOx1), which serves as an alternative to the human Ad vector and does not confer any immunity on humans, Oxford/AstraZeneca could overcome this drawback[235,236].

Moreover, Sadoff *et al*[231] revealed that a single-shot Janssen vaccination prevents severe SARS-CoV-2 infections. A total of 43783 seronegative volunteers participated in this study, and they were separated into two age groups: Group 1 (18-59 years old) and group 2 (≥ 60 years old). These participants were randomly divided into two groups of like-minded individuals in a 1:1 ratio, one receiving the placebo and the other the vaccination. The study group collected 468 confirmed cases after receiving the vaccination for 14 d. A total of 464 cases, including 116 from the vaccination group and 348 from the placebo group, were mild to moderate in severity, indicating an effectiveness of 66.9%. More than 66 moderates to severe-critical cases were confirmed to belong to the vaccine group after 28 d of follow-up, compared to 193 cases that belonged to the placebo group. Moreover, less severe-critical cases were observed among older patients than younger patients, suggesting possible early protection from the vaccine, especially in the elderly. The effectiveness of the immunization was equal across all age groups after 28 d[233].

Sinopharm COVID-19 vaccine (Sinovac)

At least five distinct COVID-19 vaccines, including conventional inactivated viral vaccines and vaccines based on an adenovirus vector, have been created and given the go-ahead for use in China. The safety and efficacy of the majority have not been extensively reported. As part of its international COVID-19 immunization global project known as COVAX®, the WHO has authorized two vaccines, the Sinopharm, Beijing, and Sinovac Corona Vac vaccines, both traditional inactivated viral vaccines, are essential to China's ambition to immunize most of its inhabitants by 2022[237]. After two dosages, the efficacy rates in clinical trials examining their safety and effectiveness from various regions of the world range from 50% to 91%. Other nations use these vaccinations, including Russia, Turkey, Brazil, Chile, Argentina, Peru, Mexico, Egypt, the UAE, Jordan, Morocco, Indonesia, and Pakistan. Although the range and incidence of adverse effects following the Sinopharm and Sinovac COVID-19 vaccinations are not documented, the methods of manufacture would imply that these vaccines are generally safe and unlikely to cause hepatocellular damage[222,238,239].

Patients with CLDs are particularly susceptible groups to increase the risk of death and more severe types of COVID-19. Many procedures or treatments for this demographic were postponed due to hospital overcrowding or to avoid putting patients at further risk. This population requires specific attention due to their underlying condition. Therefore, for these patients, immunization should also be a top priority. Interestingly, vaccination appears to be safe in stable CLDs[224]. Additionally, immunization priority was given to the high-risk liver disease such decompensated cirrhosis, liver cancer, and liver transplant recipients. They should receive the vaccination faster when their scores are higher. Indeed, the severity of the immune response induced by vaccine in these participants is unknown, and it is anticipated that it will be insufficient given their underlying illnesses and treatments. The mRNA COVID-19 vaccines are especially remarkable since they are expected to have favorable, safe, and effective characteristics in these individuals[240]. Accordingly, to get COVID-19 vaccinations, patients with CLDs receiving medical care do not need to cease their medication. Besides, patients with

HCC receiving systemic or locoregional therapy can get the vaccine without interrupting their medical care. Nevertheless, immunization should be postponed until the situation is stabilized in recent disease or fever cases. Intriguingly, immune-related adverse events are a potential outcome of vaccination interactions with immune checkpoint inhibitors, which raises concerns about their usage in patients with certain liver disorders (such as HCC) and calls for more research[241]. Influenza and pneumococcal vaccines are recommended for patients with advanced liver disorders to avoid lower immunogenicity in liver disease patients[242,243].

Despite the lack of long-term safety evidence about liver diseases patients vaccinated by SARS-CoV-2 vaccines, it is crucial to balance the potential benefits of vaccination against any possible risks, especially considering the catastrophic implications of SARS-CoV-2 infection in at-risk groups. When new vaccines are introduced, evaluation of safety and immunological response to immunization in individuals with liver disease should be conducted[244]. National and international perspective registries should start as quickly as possible, ideally without governmental obstacles. Individuals at risk should prioritize SARS-CoV-2 infection prevention by vaccination, given the promising short-term safety results of the recently approved vaccines[245].

CONCLUSION

Hepatic injuries have been approved to be associated with SARS-CoV-2 infection. Indeed, several factors have been embedded in the pathological forms of SARS-CoV-2 hepatic injuries, including viral tropism, cytokine storm, hypoxia, endothelial cells, and even some drugs that treat COVID-19. In addition, previous studies have proved that pregnant women and neonates with hepatic illness are risky for COVID-19 complications. Due to the fast spread of new SARS-CoV-2 strains, vaccines were administered and developed accordingly. In the present review, we believe that patients with CLDs especially those have severe cirrhosis, liver decompensation, and hepatobiliary cancer should be given a priority to get SARS-CoV-2 immunization. Since it is unknown whether vaccination gives sterilizing immunity and inhibits transmission from asymptomatic patients, preventative measures, such as wearing masks, proper hand washing, and social seclusion, remain of utmost relevance.

FOOTNOTES

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Country/Territory of origin: Egypt

ORCID number: Fares E M Ali 0000-0001-8341-7458; Mostafa K Abd El-Aziz 0000-0003-0252-5065; Mahmoud M Ali 0000-0001-6173-1876; Osama M Ghogar 0000-0002-2003-1408.

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REVIEW

Seronegative spondyloarthropathy-associated inflammatory bowel disease

Chrong-Reen Wang, Hung-Wen Tsai

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Chrong-Reen Wang, Department of Internal Medicine, National Cheng Kung University Hospital, Tainan 70403, Taiwan

Hung-Wen Tsai, Department of Pathology, National Cheng Kung University Hospital, Tainan 70403, Taiwan

Corresponding author: Chrong-Reen Wang, MD, PhD, Full Professor, Internal Medicine, National Cheng Kung University Hospital, No. 138 Sheng-Li Road, Tainan 70403, Taiwan.
wangcr@mail.ncku.edu.tw

Abstract

Seronegative spondyloarthropathy (SpA) usually starts in the third decade of life with negative rheumatoid factor, human leukocyte antigen-B27 genetic marker and clinical features of spinal and peripheral arthritis, dactylitis, enthesitis and extra-articular manifestations (EAMs). Cases can be classified as ankylosing spondylitis, psoriatic arthritis, reactive arthritis, enteropathic arthritis, or juvenile-onset spondyloarthritis. Joint and gut inflammation is intricately linked in SpA and inflammatory bowel disease (IBD), with shared genetic and immunopathogenic mechanisms. IBD is a common EAM in SpA patients, while extraintestinal manifestations in IBD patients mostly affect the joints. Although individual protocols are available for the management of each disease, the standard therapeutic guidelines of SpA-associated IBD patients remain to be established. Nonsteroidal anti-inflammatory drugs are recommended as initial therapy of peripheral and axial SpA, whereas their use is controversial in IBD due to associated disease flares. Conventional disease-modifying anti-rheumatic drugs are beneficial for peripheral arthritis but ineffective for axial SpA or IBD therapy. Anti-tumor necrosis factor monoclonal antibodies are effective medications with indicated use in SpA and IBD, and a drug of choice for treating SpA-associated IBD. Janus kinase inhibitors, approved for treating SpA and ulcerative colitis, are promising therapeutics in SpA coexistent with ulcerative colitis. A tight collaboration between gastroenterologists and rheumatologists with mutual referral from early accurate diagnosis to appropriately prompt therapy is required in this complex clinical scenario.

Key Words: Seronegative spondyloarthropathy; Inflammatory bowel disease; Biologics; Anti-tumor necrosis factor monoclonal antibody; Small molecules; Janus kinases inhibitor

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Core Tip: Seronegative spondyloarthropathy (SpA) with negative rheumatoid factor has spinal and peripheral arthritis, dactylitis, enthesitis and extra-articular manifestations (EAMs). It can be classified into ankylosing spondylitis, psoriatic arthritis, reactive arthritis, enteropathic arthritis, and juvenile-onset spondyloarthritis. Inflammatory bowel disease (IBD) is a common EAM in SpA, whereas extraintestinal manifestations in IBD mostly affect the joints. Anti-tumor necrosis factor monoclonal antibodies are effective medications with indicated use in SpA and IBD, a drug of choice for treating SpA-associated IBD. A tight collaboration between gastroenterologists and rheumatologists with mutual referral from early accurate diagnosis to prompt therapy is required in this complex clinical scenario.

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INTRODUCTION

Spondyloarthropathy (SpA) usually starts in the third decade of life with a shared genetic marker human leukocyte antigen (HLA)-B27 and clinical features including spinal and peripheral arthritis, dactylitis (sausage-like swelling of the digits), enthesitis (inflammation at the attachment of tendons/ligaments and joints), tenosynovitis, and extra-articular manifestations (EAMs) mostly involving the eyes, intestine, and skin[1,2]. The prevalence of SpA in population-based studies from North America is estimated to be between 0.4% and 1.3%[3]. SpA is classified as axial type with or without radiographic sacroiliitis, predominantly involving the spine, and peripheral type with or without psoriasis (PsO), inflammatory bowel disease (IBD) or preceding infection, predominantly affecting the extremities[1,2,4]. The concept of seronegative SpA, established in 1974[5], describes a group of chronic arthritis patients who have negative rheumatoid factor and classically includes ankylosing spondylitis (AS), psoriatic arthritis (PsA), reactive arthritis (ReA), enteropathic arthritis (EnA), juvenile-onset SpA (JSpA), and undifferentiated SpA (USpA)[1,6-8]. Regarding the occurrences of disease, AS is the most common types of SpA, followed by PsA, ReA, EnA, and JSpA[3]. In particular, USpA is used to describe seronegative SpA patients with suggestive features but not fulfilling the diagnostic or classification criteria for any of the currently established aforementioned other five subtypes[9]. Table 1 demonstrates demographic, clinical, laboratory, therapeutic and prognostic characters of five seronegative SpA subtypes, including AS, PsA, ReA, EnA, and JSpA.

IBDs, mainly Crohn's disease (CD) and ulcerative colitis (UC), are chronic idiopathic inflammatory disorders of the intestinal tract with progressive disease course[10-12]. CD features chronic granulomatous transmural inflammation with discontinuous lesions involving any part of the intestine, ileum, and colon in particular, complicated by intestinal granuloma, obstruction, stricture, and fistula[11], whereas UC is characterized by continuous mucosal inflammation extending from the rectum toward the colon without the above complications[12]. Extraintestinal manifestations (EIMs) occur in 25% to 40% of IBD patients and mostly affect the joints, followed by the skin, eyes, and hepatobiliary tract[10,13]. Primary sclerosing cholangitis (PSC) is the most frequently observed hepatobiliary manifestation [13,14]. IBD has been identified in 60% to 80% of PSC patients. Up to 5% of UC patients have PSC, while it is less frequent in CD patients. Furthermore, CD and UC patients have an increased risk of intestinal malignancies, such as colorectal cancer[10,15]. IBD was initially thought to be a rare disease in Asia, contrary to the West[16]. Recent population-based data have revealed a rapidly rising incidence in eastern countries while plateauing or even declining in western nations[17]. The epidemiological evolution in IBD is supposedly linked to the Westernized lifestyle and industrialization, including dietary changes, antibiotics use, hygienic status, microbial exposure and pollution, as all are potential environmental risk factors. Furthermore, increased disease awareness, advances in diagnosis, and improved healthcare access can also contribute to the increasing trend of IBD incidence[17,18]. Table 2 shows the demographic, clinical, laboratory, therapeutic, and prognostic characters of the two main types of IBD.

An individual susceptibility to IBD is strongly conditioned by the interaction between intestinal microbiota and the host immune response[19]. Westernized lifestyle-associated dysbiosis, an individual loss of diversity in microbiome composition, has been observed in IBD, and there is a trend toward restored intestinal eubiosis in such patients responding to anti-tumor necrosis factor (TNF) therapy[20]. Accumulating evidence indicates that intestinal inflammation is linked to dysbiosis occurring in rheumatic diseases[21]. The interaction between dysbiosis and the intestinal immune system can lead to

Table 1 Demographic, clinical, laboratory, therapeutic, and prognostic profiles in five seronegative spondyloarthropathy subgroups

Category	AS	PsA	ReA	EnA	JSpA
Demographic					
Sex, M:F	3:1	1:1	5-10:1	1:1	ERA 3:1, JPsA 1:2
Age, yr	20-40	35-45	Any	20-40	< 16
Laboratory					
HLA-B27	> 90%	Axial 50%-70%	60%-80%	Axial 50%-70%	ERA 40%-70%
		Peripheral 20%		Peripheral 20%	JPsA 10%
Clinical					
Affected joints	Spine, sacroiliitis	Any area	Peripheral, sacroiliitis	Peripheral	Peripheral, sacroiliitis
Peripheral	30%, lower	Common, upper	Common, lower	Common, lower	Common, lower
Sacroiliitis	100%	50%	30% in urogenital	20%	40%-60% in ERA
Dactylitis	Uncommon	Common	Common	Uncommon	20% in JPsA
Enthesitis	Common	Common	Common	Uncommon	Uncommon
EAM common	Intestine, skin, uveitis	Intestine, skin, uveitis	Skin, uveitis	Intestine, skin, uveitis	Intestine, skin, uveitis
Treatment	Spinal physical therapy, NSAIDs/cDMARDs for peripheral SpA, biologics, JAKi	NSAIDs, avoid CS, cDMARDs for peripheral SpA, biologics, JAKi, PDE4i	NSAIDs, antibiotics for chlamydia-induced ReA, cDMARDs for peripheral SpA	Coxibs/cDMARDs for peripheral SpA, biologics, JAKi	Spinal physical therapy, NSAIDs/cDMARDs for peripheral SpA, biologics
Prognosis	Life-threatening EAMs with heart, intestine or neurological involvement	Comorbidities associated with more severe disease activity	Usually a self-limited disease	Rarely grave EnA in controlled intestinal activity	More spinal deformity and THR as compared with adult SpA or other JIA subtypes

AS: Ankylosing spondylitis; cDMARD: Conventional synthetic disease-modifying anti-rheumatic drug; Coxib: Cyclooxygenase-2 inhibitor; CS: Corticosteroid; EAM: Extra-articular manifestation; EnA: Enteropathic arthritis; ERA: Enthesitis-related; F: Female; IBD: Inflammatory bowel disease; JAKi: Janus kinase inhibitor; JPsA: Juvenile psoriatic arthritis; M: Male; NSAID: Non-steroidal anti-inflammatory drug; PDE4i: Phosphodiesterase 4 inhibitor; PsA: Psoriatic arthritis; ReA: Reactive arthritis; SpA: Spondyloarthropathy; JSpA: Juvenile-onset spondyloarthropathy; THR: Total hip replacement.

the aberrant activation of immune cells that can recirculate from the gut to the EIM sites as observed in SpA[19,21]. Subclinical gut inflammation in SpA patients represents the repertoire in which immune cells are activated, and is correlated with the severity of spinal inflammation[22]. Genetic risk factors are shared between SpA and IBD, and changes in the composition of the intestinal microbiota are observed in both diseases, indicating that joint and gut inflammation is intricately linked in SpA[19,23].

Since SpA and IBD patients share common genetic and immunopathogenic mechanisms[23], SpA patients have an up to four-fold increased risk of IBD compared to the general population. Different forms of SpA can be associated with variable frequencies of intestinal involvement, whereas articular involvement is frequently observed in IBD. Nevertheless, the chronic medication history of patients' needs to be considered to appropriately evaluate gastrointestinal symptoms in SpA. In addition to direct gastrointestinal adverse reactions, it is necessary to rule out infectious complications with a detailed microbiological survey due to potential immunosuppressive effects. Furthermore, it has been suggested that SpA patients should be evaluated by gastroenterologists when suspected IBD symptoms are present, including rectal bleeding, perianal disease, and chronic diarrhea with organic characteristics [24]. Although individual protocols for managing each disease are available, the standard therapeutic guidelines of seronegative SpA-associated IBD patients remain to be established. In particular, some therapeutic options used to manage one disease might have a negative impact on another disease[25].

Herein, we provide a thorough overview on coexisting IBD in different subtypes of seronegative SpA patients.

ANKYLOSING SPONDYLITIS

AS is a chronic autoimmune disease mainly involving spinal and sacroiliac as well as peripheral joints, with up to 50% of cases mainly affecting the hips and knees[1,2]. There is a similar pooled prevalence of 0.25% and 0.20% in AS from Caucasian-dominant Europe and North America, respectively[1,26]. Furthermore, this disorder has a prevalence of 0.25% and 0.20% in Taiwan and China, respectively, both

Table 2 Demographic, clinical, laboratory, therapeutic, and prognostic profiles in two main types of inflammatory bowel disease

Category	Ulcerative colitis	Crohn's disease
Demographic		
Sex, M:F	1:1	1:1
Age at onset in yr	30-50	10-40
Laboratory		
ANCA	Common	Rare
ASCA	Rare	Common
Clinical		
Origin/Location	Rectum/colon, rectum	Terminal ileum/any part
Distribution	Continuous	Skip lesions
Pathology		
Inflamed thickness	Mucosa, submucosa	Transmural
Crypt abscess	Common	Uncommon
Granuloma	Rare	Common
Fissure	Uncommon	Common
Fibrosis	Rare	Common
Treatment	ASA, CS, IS, biologics, JAKi, S1PR modulator, surgery for refractory medical disease or malignancy	CS, IS, biologics, surgery for refractory medical disease, complication or malignancy
Prognosis	Complete remission in most patients, low surgical requirement	Prolonged remission in about 20% of patients, 10-yr surgical resection risk near 50%

ANCA: Anti-neutrophil cytoplasmic antibody; ASA: Aminosalicylate; ASCA: Anti-*Saccharomyces cerevisiae* antibody; CS: Corticosteroid; IS: Immunosuppressant; JAKi: Janus kinase inhibitor; SIPR: Sphingosine-1-phosphate receptor.

with a Han Chinese-dominant population[27,28]. In the EAMs of AS patients, frequencies of about 30% have been found for anterior uveitis in both Caucasian and Han Chinese populations[29,30]. Typical attacks are abrupt and unilateral, with pain, photophobia and visual impairment, frequently alternating from one eye to another[1,2]. PsO occurs in more than 10% of Caucasians, more common than in Han Chinese patients[1,29]. There is a 5% to 10% incidence of IBD in AS patients from western countries[29], whereas frequencies of only 0.4% to 0.6% have been identified for IBD in Han Chinese AS populations [30,31]. In comparison with earlier years, there is a sharply increasing current incidence of IBD, without changes in AS prevalence from East Asia[16]. Despite a progressively narrowing gap between Asia and West, the prevalence of IBD remains much higher in Western countries compared to that in Asian nations. In the 21st century, the pooled prevalence of IBD in North America and Europe is estimated to be about 0.3% of the general population[17,18], whereas in the Han Chinese population, the recent prevalence of UC and CD per 100000 individuals has risen to 12.8 and 3.9 in Taiwan and 24.5 and 18.6 in Hong Kong, respectively[32,33]. Although genome-wide association studies have demonstrated shared risk alleles between the two disorders, the above-mentioned clinical observations suggest that ethnicity can be an important factor causing inconsistency in the coexistent frequencies of IBD in AS between Caucasian and Han Chinese populations. Further investigations in the gut-joint axis of inflammation in SpA should consider the issue of disconnection between the occurrence of IBD in AS on the basis of ethnicity, *i.e.*, Han Chinese or other races[23].

In addition to pharmacotherapy, physical therapy and regular exercise in AS patients, either with active or stable axial SpA, can improve the symptoms and functions by maintaining posture and spinal flexibility[34]. Nonsteroidal anti-inflammatory drugs (NSAIDs) are the drug of choice for initial therapy for axial SpA. There are frustrated outcomes regarding axial symptoms, spinal pain in particular, in AS patients receiving conventional disease-modifying anti-rheumatic drug (cDMARD) therapy, including methotrexate (MTX) and sulfasalazine (SAZ). Nevertheless, clinical evidence supports the use of cDMARDs for controlling peripheral arthritis in AS patients. With advances in the understanding of immunopathogenesis in AS[1,2,23], there are increasing numbers of novel medications, including biologics targeting TNF or interleukin (IL)-17 and small-molecule agents, Janus kinase inhibitors (JAKis) [34]. Such therapies have been associated with substantial improvements in disease activity and quality of life.

IBD manifestations in AS represent a clinical challenge by increasing the disease burden with difficulties in managing such patients[35]. Nevertheless, the introduction of new therapeutics targeting both articular and intestinal manifestations, TNF inhibitor (TNFi) in particular, has revolutionized the treatment of patients not responding to conventional medications[1,2,36,37]. In Table 3, the English-language literature is summarized for published reports related to the occurrences of IBD, flare-up or new-onset, in AS patients under the treatment of different TNF blockades, including adalimumab (ADA), certolizumab pegol (CZP), etanercept (ETA), golimumab (GOL), and infliximab (IFX)[38-62]. Notably, most of the enrolled cases were predominantly Caucasian. Since the dosages of TNFi for IBD therapy are higher than those used in AS, new-onset or flare events of IBD can occur in such patients during the therapeutic period, indicating the potential inefficacy of particular TNF blockade in the AS-associated IBD manifestation. Notably, monoclonal antibodies (mAbs) have better protective effects than recombinant soluble TNF receptor fusion proteins. Despite the lack of observed IBD events in AS patients during three GLO randomized clinical trials (RCTs), four cases were reported to have a flare at 2 mo to 5 mo after starting treatment[63].

Table 4 shows the demographic, clinical, laboratory, medication, course and outcome profiles in 4 AS-associated IBD patients, 3 UC patients, and 1 ulcerative proctitis (UP) patient with moderate to severe activity. All received endoscopic biopsy with characteristic histopathological changes (Figure 1A, B, G, and H). This 5-year observation enrolled 878 (86% male) Han Chinese AS patients by the authors. There was a 0.5% occurrence of IBD. At IBD onset, there was a long disease period (12 years to 25 years, 16.5 ± 5.8) with high-activity treated with NSAIDs and cDMARDs. For IBD therapy, corticosteroids (CSs) were prescribed in the acute stage with topical and systemic high-dose for case No. 1 and others, respectively, followed by aminosalicylate (ASA) or plus low-dose CS for maintenance. Nevertheless, all experienced a disease relapse, while case No. 3 had colonic perforation that required surgical intervention. Repeated endoscopic biopsy in case No. 1 showed chronic active rectitis (Figure 1C and D). Due to refractory activity, all started ADA injection with 40 mg biweekly. A relapse occurred in case No. 2 under the tapered dosage of 40 mg every 4 wk (Figure 1I and J); however, there were no more flares for 4.8 years after resuming a biweekly regimen. Altogether, all had no IBD flares under ADA 40 mg biweekly injection without CS, cDMARD, or immunosuppressants for 4.3 years to 5.8 years (5.1 ± 0.7). All had clinical IBD remission and only mild non-specific lymphocytic infiltration (Figure 1E, F, K, and L). Despite histopathological changes more resistant to resolution than clinical remission in IBD[64], whether microscopic healing provides additional outcome benefits remains to be determined.

Reactivation or development of IBD in AS patients receiving ETA therapy is thought to be caused by particular structure, administration mode, neutralizing effect, and/or pharmacokinetic characteristics of ETA[65]. Despite the indirect evidence based on the risks of IBD among AS patients during biologics therapy, the American College of Rheumatology (ACR)/Spondylitis Association of America/Spondyloarthritis Research and Treatment Network have recommended the treatment with anti-TNF mAbs over other biologics in adults with AS and coexisting IBD[66]. Notably, AS patients under IL-17 blockade therapy have increased risks of IBD development or exacerbation compared with the placebo-controlled group[67,68]. Moreover, according to the management recommendations of axial SpA from Assessment of Spondyloarthritis International Society/European League Against Rheumatism for the EAMs, anti-TNF mAbs are effective in IBD therapy and in the prevention of uveitis recurrence, whereas ETA has no effects on treating IBD and contradictory outcomes in uveitis prevention[69]. The use of a special mAb can be made in consultation with gastroenterologists due to different indications of mAbs in the IBD subtype, ADA and IFX for CD or UC, CZP for CD, and GOL for UC.

In the Han Chinese population, ADA is an effective biologic agent in controlling the articular activities in AS[70]. For ADA therapy in IBD, higher remission and response rates have been observed in China compared with those in Western countries[71]. Furthermore, its efficacy has also been demonstrated in moderate to severe IBD patients in Taiwan with more rigorous prescription criteria than in the West[72]. Interestingly, contradictory to our favorable therapeutic results without any UC flares in Han Chinese AS-associated IBD patients (Table 4), in an RCT of AS patients (97% Caucasian) under 40 mg ADA biweekly injection for 24 wk, 2 cases experienced a UC flare, 1.9 events per 100 patient-years *vs* none in the placebo group[54]. In systemic rheumatic disorders, clinical outcomes under the similar immunosuppressant treatment can be variable in different racial populations[73], while the ethnic factor has been considered to be involved in therapeutic responses to biologics therapy[74]. Further international collaborations in large-scale RCTs enrolling more ethnic groups might be needed to evaluate such an issue in AS-associated IBD.

UP patients with inflammation limited in the rectum can manifest as tenesmus, urgency, and rectal bleeding[75]. Such patients might fail to improve and require additional medications despite the beneficent effects of ASA and CS. Medical therapy in UP refractory to the standard treatment is challenging due to no evidence-based large-scale data of other medications[76]. In addition, UC patients limited to the rectum are usually excluded from the RCT on biologics therapy. Nevertheless, a recent referral cohort with 118 cases followed for up to 20 years revealed that UP resistant to conventional therapies could have clinical responses to anti-TNF mAbs[77]. Furthermore, long-term outcome in UP patients receiving biologics therapy was superior to azathioprine treatment, consistent with the results demonstrating beneficent efficacy of refractory UP under anti-TNF therapy from a retrospective cohort with 104 cases[77,78]. In our 5-year observation, a UP case (No. 1 in Table 4) resistant to ASA use

Table 3 Inflammatory bowel disease manifestation in ankylosing spondylitis patients receiving approved tumor necrosis factor inhibitor or Janus kinase inhibitor therapy published in the English literature

No.	Clinical trials, *n*	Countries involved in clinical trials	Cases, *n*	TNFi or JAKi	IBD manifestation events, flare-up and new-onset	IBD manifestation events per 100 patient-yr[1]	Ref.
1	7	Canada, Germany, Netherlands	366	IFX	1 CD	0.2	[38-44]
2	9	European nations, United Kingdom, United States	724	ETA	14 (8 CD, 6 UC)	2.0	[45-52]
3	5	France, Germany, Netherlands, United States, *etc.*	2026	ADA	14	0.7	[53-55]
4	3	Canada, Germany, Netherlands, United States, *etc.*	837	GOL	0	0	[56-58]
5	1	Belgium, Canada, France, Germany, Netherlands, United States	121	CZP	1 CD	0.2	[59,60]
6	1	Australia, Canada, European nations, United States, *etc.*	133	TOF	0	0	[61]
7	1	Australia, Canada, European nations, Israel, United States, *etc.*	211	UPA	1 CD	1.8	[62]

[1]One point six events per 100 patient-years in placebo groups by pooling 1015 ankylosing spondylitis patients under clinical trials[38,42,45,46,47,49,52,54,56,59,61,62].

ADA: Adalimumab; AS; Ankylosing spondylitis; CD: Crohn's disease; CZP: Certolizumab pegol; ETA: Etanercept; GOL: Golimumab; IBD: Inflammatory bowel disease; IFX: Infliximab; JAKi: Janus kinase inhibitor; No.: Number; Ref.: Reference; TNFi: Tumor necrosis factor inhibitor; TOF: Tofacitinib; UC: Ulcerative colitis; UPA: Upadacitinib.

showed a clinical remission under ADA therapy for more than 4 years.

A better understanding of the complex IBD pathogenesis has brought about a therapeutic approach focusing on clinical and histopathological remission with precise molecular targeting of inflammatory cascades. Since the successful results on the use of IFX in CD patients in 1997, three additional anti-TNF mAbs, two anti-integrin mAbs, three small-molecule agents including a sphingosine-1-phosphate receptor modulator and two JAKis, and two mAbs targeting the p40 subunit of IL-12/IL-23 and the p19 unit of IL-23 have been approved by the United States Food and Drug Administration (FDA), expanding the options for IBD treatment[79].

The signaling pathway of JAK-signal transducer and activator of transcription (STAT), including JAKs 1-3, STATs 1-6, and tyrosine kinase 2, can regulate miscellaneous cytokine receptors and has pathogenic roles in various autoimmune and inflammatory disorders[80]. Furthermore, individual cytokine receptors can recruit their own combined JAKs and STATs to activate distinct processes in different targeted cells, while antagonizing a specific JAK can inhibit diverse cytokine pathway, expanding the effects of JAKi on cytokine-targeted therapy[81]. Tofacitinib (TOF), a pan-JAKi targeting JAKs 1-3, and upadacitinib (UPA), a selective JAK1 inhibitor, have been approved by the FDA in adult UC with moderately to severely activity with intolerance or poor responses to TNF mAbs in 2018 and 2022, respectively, overcoming the challenges of using biologics to avoid immunogenicity induction and parenteral administration[79]. Furthermore, TOF and UPA have received an indication in adult AS with an inadequate response or intolerance to TNFi in 2021 and 2022, respectively (Table 3). In a recent phase III RCT enrolling 136 AS patients with more than 80% Caucasians, there were no observed IBD events under TOF 5 mg bid therapy for 16 wk[61], validating its expected effects for UC manifestation in TNFi-refractory AS patients. Nevertheless, there was an observed new-onset CD event under UPA 15 mg once daily treatment for 14 wk in another phase III RCT enrolling 209 AS patients dominant in Caucasians (1.8 events per 100 patient-years)[62].

IL-12 helps naïve T cells differentiate into type 1 T helper (Th1) cells secreting IL-6, interferon (IFN)-γ and TNF, while IL-23 stimulates Th17 cells to express IL-17, IFN-γ, TNF, granulocyte-macrophage colony-stimulating factor, and IL-21, all of which promote mucosal inflammation in IBD patients[79,82]. IL-12 is encoded by two separate genes, IL-12A (p35) and IL-12B (p40), to form an active heterodimer following protein synthesis with p35 and p40 chains, while IL-12 p40 chain can dimerize with IL-23 p19 chain to form IL-23[82]. Ustekinumab (UST), a p40 chain mAb, was approved for the treatment of moderately to severely active CD patients who failed or were intolerant to treatment with anti-TNF therapy in 2016, and for moderately to severely active UC in 2019[79]. Moreover, risankizumab (RIS), a

Table 4 Demographic, clinical, laboratory, medication, course, and outcome profiles in 4 ankylosing spondylitis-associated inflammatory bowel disease patients from 2017 January to 2021 December[30]

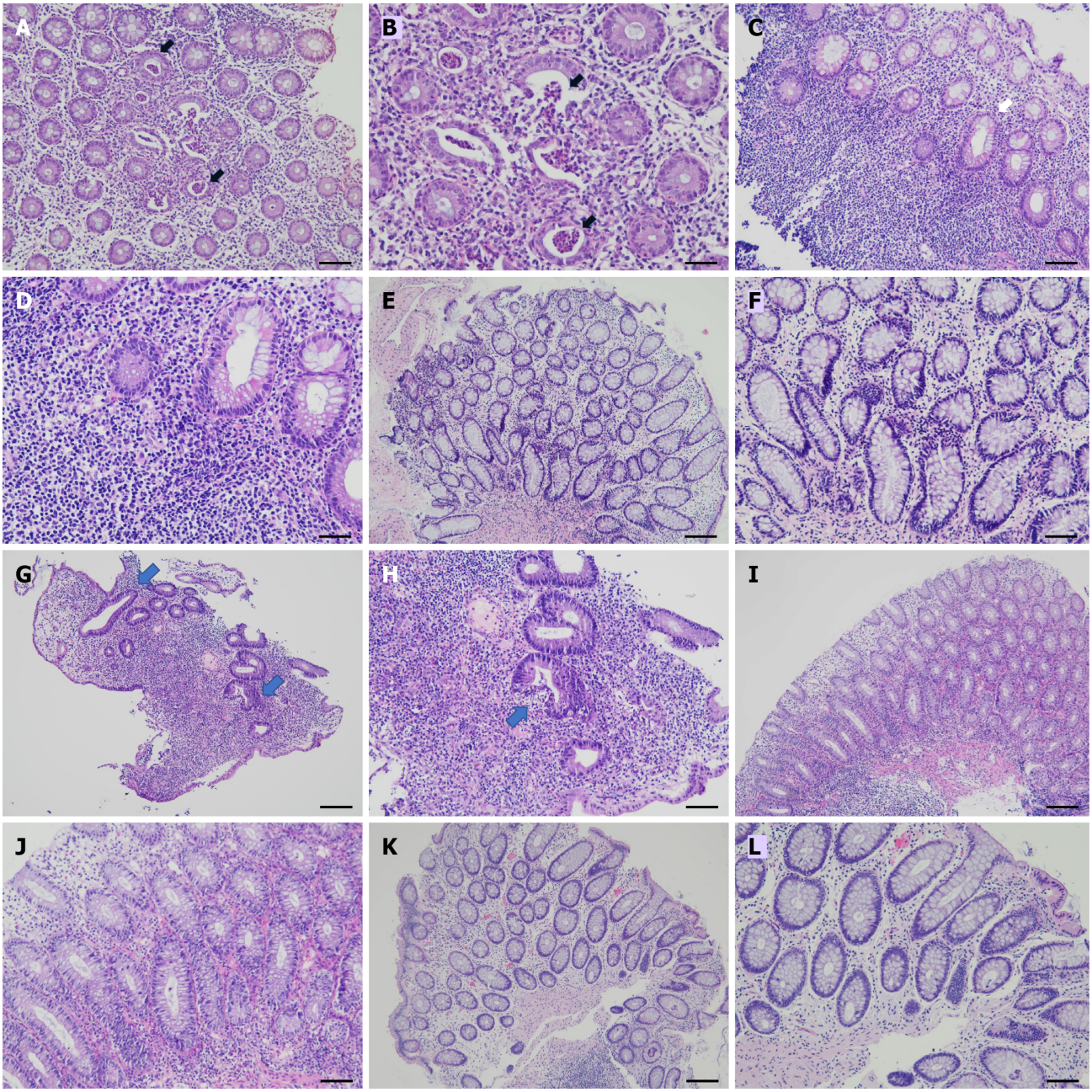
No.	Age in yr and sex	[1]AS period in yr	Affected joints	Other EA	[3]BASDAI/[2] AS medication	HLA-B27/[3] ESR	IBD clinical manifestation	IBD entity/[6] severity	[4]IBD medication	Disease course, under ADA 40 mg q2w SCI	Final outcome
1	42, F	12	SI, spine, hip	Uveitis	7.6/NSAIDs	Positive/38	Rectal bleeding, BWL, anemia	UP/ moderate, MS 9	CS, mSAZ, ADA 40 mg q2w	No IBD relapse for 4.3 yr	AS in low activity with BASDAI 2.0-2.5, IBD in remission, MS 0
2	35, M	15	SI, spine, hip	Uveitis	8.8/NSAIDs, SAZ	Positive/80	Bloody diarrhea, BWL, fever, anemia	UC/severe, MS 12	CS, mSAZ, ADA 40 mg q4 to q2w	No IBD relapse for 4.8 yr	AS in low activity with BASDAI 2.5-3.0, IBD in remission, MS 1
3	45, M	14	SI, spine, hip	Nil	8.4/NSAIDs, SAZ	Positive/42	Bloody diarrhea, BWL, anemia, [5] colon perforation	UC/severe, MS 11	CS, SAZ, ADA 40 mg q2w	No IBD relapse for 5.8 yr	AS in low activity with BASDAI 2.5-3.0, IBD in remission, MS 2
4	45, F	25	SI, spine, shoulder hip	Nil	8.1/NSAIDs, SAZ, MTX	Positive/35	Bloody diarrhea, BWL, anemia	UC/severe, MS 11	CS, SAZ, ADA 40 mg q4 to q2w	No IBD relapse for 5.3 yr	AS in low activity with BASDAI 2.5-3.0, IBD in remission, MS 1

[1]AS duration before the inflammatory bowel disease (IBD) development.
[2]Methotrexate 15 mg per wk, salazopyrin 2 to 3 g/d, nonsteroidal anti-inflammatory drugs only with Coxibs after IBD diagnosis.
[3]At disease onset of IBD.
[4]High-dose corticosteroids (CS, 1-2 mg/kg/d prednisolone equivalent doses) for acute ulcerative colitis (UC), topical CS for active ulcerative proctitis, low-dose CS for UC maintenance.
[5]Perforation at the splenic flexure, under double barrel colostomy and abdominal abscess drainage.
[6]MS: Mayo score, 11-12 severe, 6-10 moderate, 3-5 mild, 0-2 remission.
Age at diagnosis of inflammatory bowel disease. ADA: Adalimumab; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BWL: Body weight loss; CS: Corticosteroids; ESR: Erythrocyte sedimentation rate (normal value ≤ 15 mm/h); F: Female; IBD: Inflammatory bowel disease; M: Male; mSAZ: Mesalazine; MTX: Methotrexate; No.: Number; NSAIDs: Nonsteroidal anti-inflammatory drugs; q2w: Every 2 wk; SCI: Subcutaneous injection; UP: Ulcerative proctitis; SAZ: Salazopyrin; SI: Sacroiliac; UC: Ulcerative colitis; WNL: Within normal limit.

p19 chain mAb, was approved for the treatment of moderately to severely active CD patients who failed or were intolerant to treatment with TNF blockers in 2022. Nevertheless, both UST and RIS have no indication for treating TNFi-refractory AS patients. In a national cohort study evaluating the long-term UST effects in 152 CD patients including 17 associated with AS, efficacy was not identified in SpA symptoms[83].

PSORIATIC ARTHRITIS

PsA, a chronic inflammatory arthritis with impaired function and reduced quality of life, develops in up to 30% of PsO patients[84]. Both axial and peripheral joints can be involved with five clinical patterns not mutually exclusive, including the most commonly observed asymmetric oligoarticular, symmetric polyarticular, distal interphalangeal joint-predominant, axial/SpA-predominant, and the rarely identified deforming/destructive subtype, *i.e.*, arthritis mutilans[4,84]. Cutaneous lesions can be found in most cases at the time of articular presentation; however, in up to 15% of PsA patients, arthritis can antedate the appearance of skin disease, *i.e.*, PsA sine PsO[85]. Dactylitis or enthesitis has been reported in up to 50% of patients. In addition, about 40% to 50% of patients are positive for HLA-B27, higher in axial than the peripheral-only type[84]. The prevalence of PsA is between 0.3% and 1.0% in the United States[84], whereas it is much lower in Han Chinese, with the prevalence ranging from 0.01% to 0.1%[86, 87].



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Figure 1 Serial histopathological findings of ulcerative proctitis before and after therapy from rectum biopsy specimens. Hematoxylin and eosin stain. A and B: Rectal mucosa before therapy shows acute rectitis with neutrophilic infiltrates and crypt abscess in case No. 1 (arrows), 100 × (A) and 200 × (B); C and D: Rectal mucosa shows features of chronicity including dense lymphocytic infiltration, basal lymphoplasmacytosis and crypt distortion in case No. 1 (arrow), 100 × (C) and 200 × (D); E and F: Rectal mucosa after adalimumab therapy shows mild non-specific lymphocytic infiltration in case No. 1, 100 × (E) and 200 × (F); G and H: Colonic mucosa before therapy shows crypt distortion (arrows) and lymphoplasmacytic infiltration in the lamina propria in case No. 2, 100 × (G). The crypt shows distortion and neutrophilic infiltration (arrow), 200 × (H); I and J: Colonic mucosa shows less crypt distortion and lymphoplasmacytic infiltration as compared with (G and H) before adalimumab (ADA) therapy in case No. 2, 100 × (I) and 200 × (J); K and L: Colonic mucosa after ADA 40 mg injection once every 2 wk shows mild non-specific lymphocytic infiltration in case No. 2, 100 × (K) and 200 × (L). Bars shown on 100 × and 200 × photomicrographs correspond to 100 μm and 50 μm, respectively.

Uveitis has been identified in 8% of PsA patients, affecting the anterior and posterior poles of the eyes [84]. Comorbidities in PsA are associated with more severe disease activities, including diabetes, hypertension, hyperlipidemia, metabolic syndrome and fatty liver, while there is an increased risk of cardiovascular events[88]. It is estimated that 9.6% of CD patients have PsO (2.2% in the general population), while 0.5% of PsO patients have CD (0.2% in general population)[89]. Despite there being a lower occurrence than in CD, there is a similar trend between patients with UC and PsO[90]. In comparison with patients with PsO alone, patients with PsA have a higher risk of IBD coexistence[91]. Cohort studies have demonstrated an increased risk of concomitant CD[92] or UC[93] in PsA patients. Furthermore, IBD is more common in PsA patients with greater activities, and in the axial than the

peripheral-only subtype[94].

Based on high-quality, evidence-based, domain-focused recommendations for medication selection in PsA, the Group for Research and Assessment of Psoriasis and Psoriatic Arthritis updated treatment recommendations for such patients in 2021[95]. Choice of therapy for an individual patient should ideally address all active disease domains, related EAMs, and comorbidities. For patients with axial involvement not responding to NSAID use, initiation of a targeted therapy is strongly recommended, including TNFi, IL-17i, and JAKi. For peripheral arthritis, cDMARDs such as MTX and SAZ can be used as first-line therapy. In dactylitis, enthesitis, nail or topicals-unresponsive PsO, cDMARD-refractory peripheral arthritis, recent evidence supports the use of IL17i (ixekizumab, SEK), IL23i (guselkumab, RIS), JAKi (TOF, UPA), phosphodiesterase 4 inhibitors (PDE4is, apremilast), TNFi (ADA, CZP, ETA, GOL, IFX), and UST. For PsA-related EAMs, MTX or TNF mAbs can be used for the treatment of anterior uveitis. TNF mAbs and UST have demonstrated their therapeutic efficacy in CD and UC. TOF and UPA are effective in treating UC, while RIS has efficacy in CD therapy. Notably, IL17i can increase the risk of IBD onset or exacerbation, and their use should be avoided in IBD, even in disease remission [25,96]. Since comorbidities are associated with greater PsA activity and reduced therapeutic responses, their recognition and monitoring with appropriate management is important for health-care providers caring for such patients[91,95].

REACTIVE ARTHRITIS

ReA has a sterile, transient nature, typically with an asymmetric oligoarthritis of the lower limbs following a preceding genitourinary or gastrointestinal tract infection, ranging from several days to weeks [97-99]. Depending on the causative agents and other factors, after more than 6 mo, about one-quarter of patients can progress into chronic arthritis requiring long-term therapy. This disease shares the overlapping features of seronegative SpA, including HLA-B27 association, axial involvement, sacroiliitis, enthesitis, dactylitis, and EAMs. It is an uncommon disease that occurs in young adults, with a global prevalence of 0.02% to 0.04%[86,97-99]. Post-venereal ReA most commonly affects men, while post-enteric ReA affects men and women equally. Genital *Chlamydia trachomatis* is the most common cause of ReA, and other common responsible enteric strains include *Yersinia*, *Salmonella*, *Shigella*, and *Campylobacter*. Similar to human immunodeficiency virus-induced ReA, the development of ReA has been identified in post-coronavirus disease 2019 (COVID-19) illness, with negative results of synovial COVID-19 polymerase chain reaction test[99].

Eye involvement including anterior uveitis and conjunctivitis preceding arthritis occurs in one-fifth of patients[100], while up to half of cases have mucocutaneous lesions with characteristic keratoderma blennorhagicum and circinate balanitis[97-99]. Upon colonoscopic biopsies of the terminal ileum and colon, histological alterations mimicking IBD with the features of acute enterocolitis or early CD were found in two-thirds of ReA patients despite an asymptomatic condition in most cases[101]. Notably, there are no known reports of increased IBD occurrences in ReA patients.

Although the disease course of post-dysentery ReA is unaltered by antibiotics use, such therapy is indicated for the identification of *C. trachomatis* infection[98,99]. Due to a self-limited nature in most ReA patients, NSAIDs are prescribed as first-line therapy. In patients not responding to NSAIDs or with chronic ReA, cDMARDs are indicated with SAZ as the drug of choice and MTX as an alternative. In patients refractory to cDMARD treatment, off-label use of ETA has shown beneficial effects[102].

ENTEROPATHIC ARTHRITIS

Musculoskeletal conditions with articular, periarticular, muscular, and skeletal manifestations are frequently observed, with an up to 50% frequency in IBD patients[103]. Rheumatological EIMs are associated with HLA-A2, DR1, and DQw5 alleles in CD, and with DRB1*0103, B27, and B58 alleles in UC[104]. Arthritis is the most common EIM in IBD involving axial (spondylitis, sacroiliitis), peripheral joints, or a combination. The prevalence of arthritis decreases with increasing age in IBD patients[105]. It occurs equally in both sexes, more commonly in CD with colon involvement than in UC, and can precede, be concomitant with, or follow the onset of IBD[106]. Peripheral arthritis can be classified into two entities: Type 1 pauciarticular and type 2 polyarticular (Table 5)[107-109]. Type 1 arthropathy is often acute, asymmetrical, and affecting less than five joints, commonly involving the large knee joint. It is usually related to IBD activity and is self-limiting, with a duration of no more than 10 wk. Treatment of the underlying intestinal inflammation is usually associated with improvement of arthritis. Type 2 arthropathy is a symmetrical arthritis involving five or more joints, commonly involving the small metacarpophalangeal joint. It is not related to IBD activity and may persist for years with articular erosion and destruction. There is an association of type 1 arthropathy with erythema nodosum, uveitis and HLA-DRB1*0103, B35 and B24, and type 2 arthropathy with uveitis and HLA-B44[108]. Notably, such a categorization of peripheral arthritis can be related more to the duration and progression of articular presentation, while the patients with a polyarticular manifestation can begin their clinical

Table 5 Classification of inflammatory bowel disease-associated peripheral arthritis

Category	Type 1 pauciarticular	Type 2 polyarticular
Prevalence	4% to 5% in IBD, higher in CD than UC	3% in IBD, higher in CD than UC
Joint manifestation		
Involved numbers	< 5	≥ 5
Articular distribution	Large joint, asymmetric	Mainly small joint
Involved area with the decreasing frequencies	Knee, ankle, wrist, elbow, MCP, hip, shoulder, MTP, PIP	MCP, knee, PIP, wrist, ankle, elbow, hip, shoulder, MTP
Erosion/destruction	Absent	Present
Clinical course	Early in IBD disease course, acute and self-limiting (mostly under 10 wk)	Arthritis for months, episodic exacerbation for yr
Disease characters		
IBD activity	Parallel with activity	Independent of activity
Other EIM	EN, uveitis	Uveitis
HLA association	HLA-B27, B35, DR*0103	HLA-B44
Treatment	Control of IBD activity, coxibs, CS, cDMARDs (SAZ 1st choice), TNF mAbs for refractory cases, JAKi for anti-TNF failure	Coxibs, CS, cDMARDs (SAZ 1st choice), TNF mAbs for refractory cases, JAKi for anti-TNF failure

CD: Crohn's disease; cDMARD: Conventional disease-modifying antirheumatic drug; Coxib: Cyclooxygenase 2 inhibitor; EIM: Extra-intestinal manifestation; EN: Erythema nodosum; HLA: Human leukocyte antigen; IBD: Inflammatory bowel disease; JAKi: Janus kinase inhibitor; mAb: Monoclonal antibody; MCP: Metacarpophalangeal; MTP: Metatarsophalangeal; PIP: Proximal interphalangeal; PsA: Psoriatic arthritis; SAZ: Salazopyrin; S1PR: Sphingosine-1-phosphate receptor; TNF: Tumor necrosis factor; UC: Ulcerative colitis.

course with an oligoarticular involvement[104].

Axial involvement can be a part of IBD but independent of gut pathology. It is more common in CD than in UC, with an up to 25% frequency[110]. Most IBD patients with axial spondylitis are HLA-B27-positive despite a lower association rate than idiopathic AS, 50% to 70% *vs* more than 90%. There is a 5% to 10% occurrence of AS in IBD with a 1:1 sex ratio and a development at any age, rather than a 3:1 male to female ratio and onset before 40 years of age in idiopathic AS[111]. Although sacroiliitis can be detected by magnetic resonance imaging (MRI) in IBD patients, most of them are asymptomatic, HLA-B27-negative and without progression into AS[112]. The prevalence of symptomatic sacroiliitis is estimated to be less than 10%.

Dactylitis, enthesitis, and tenosynovitis also occur in IBD patients as musculoskeletal EIMs. Enthesitis presenting with Achilles tendinitis, plantar fasciitis, and chest wall pain can lead to structural changes of underlying bones with functional disability[109]. Ultrasonography or MRI examination of the affected area can help in earlier detection missed by clinical inspection[113].

NSAIDs are suggested as initial therapy for peripheral and axial SpA. Nevertheless, their use is controversial in IBD due to an association with the development of intestinal ulcerations and flares of IBD[114]. Although the safety of cyclooxygenase 2 inhibitors have been investigated[115,116], their use should be limited to a short course during the IBD remission. Systemic CS can be helpful for peripheral arthritis despite ineffectivity in controlling axial SpA and enthesitis, while intra-articular CS injection may be effective in cases with limited numbers of joint involvement[106,109]. SAZ, a formulation of ASA available in intestinal therapy, is an effective cDMARD in improving peripheral arthritis, but not axial arthritis or sacroiliitis in IBD patients[117]. MTX is an alternative cDMARD recommended for the treatment of IBD-associated peripheral SpA[118]. Furthermore, TNFi can be reserved for patients with IBD-associated axial or peripheral SpA not responsive to conventional therapies[119]; however, ETA should be avoided due to its inefficacy for IBD treatment and a potential clinical exacerbation[120]. Since the doses of TNF blockade for IBD therapy is higher than those used in treating SpA, it is recommended that high-dose regimen is preferred in IBD-associated SpA during active intestinal disease[121]. Two JAKis have been approved for treating TNFi-refractory UC and AS/PsA patients. Despite the lack of an evidence-based indication, such therapy might be considered in UC-related EnA patients lacking therapeutic responses to anti-TNF therapy. Although UST use is indicated for IBD and PsA therapy, it is only effective in CD/UC-associated peripheral rather than axial SpA[122].

At least 5% of IBD patients, more frequently in CD than UC, experience ocular EIM, with uveitis as the commonest manifestation, particularly in those cases associated with arthritis[123,124]. Anterior uveitis in patients with IBD is initially treated with CS eye drops, followed by systemic CS or IS if unsuccessful[104]. Anti-TNF mAbs have shown efficacy in IBD-associated uveitis, while their use can be

considered in cases refractory to the aforementioned treatment[125].

JUVENILE-ONSET SPONDYLOARTHRITIS

JSpA, a distinct disease to adult SpA, constitutes up to one-third of juvenile idiopathic arthritis (JIA), and usually affects males and starts in early adolescence (before the age 16)[126-129]. This disease primarily affects children fulfilling the criteria for JIA categories of enthesitis-related arthritis (ERA) and juvenile psoriatic arthritis (JPsA) as well as undifferentiated arthritis with either features[127,130]. An approximate 20% prevalence of JSpA was found in JIA cohort studies[131], while approximately 10% of adult AS patients have an onset of disease in childhood[1,3]. There is HLA-B27 positivity detected in 40% to 60% of ERA, whereas only 10% of JPsA patients show HLA-B27 positivity[129]. JSpA commonly manifests with peripheral arthritis, usually asymmetric, oligoarticular, involving joints of the lower extremities including the hip, knee, ankle, and midfoot. Tender entheses are commonly present at insertions of the patellar ligament at the inferior patella, plantar fascia at the calcaneus, and the Achilles tendon[132]. About 40% to 60% of ERA cases have sacroiliitis, an early sign of axial involvement, in their disease course[129]. Nevertheless, children are known to have silent sacroiliitis without inflammatory back pain[127]. Dactylitis can be observed in 30% of patients with JPsA[133]. JSpA has a poorer outcome with more spinal deformity and need for total hip replacement, as compared with cases of other forms of JIA and their adult counterparts[127].

Similar to the adult-onset disease, common EAMs in JSpA include skin, eye, and bowel involvement [126-129]. The overall prevalence of uveitis, more common with acute anterior uveitis, is approximately 10%[134]. Two-thirds of children with SpA have been reported to have gastrointestinal symptoms[135]. Intestinal inflammation on ileocolonic biopsy has been identified in JSpA[136], while ERA with sacroiliitis had increased levels of fecal calprotectin, a gastrointestinal inflammation marker[137]. IBD in children might begin with arthritis before clinically evident intestine inflammation, while difficult-to-control arthritis, longstanding, vague gastrointestinal complaints and anemia might be helpful clues for earlier diagnosis[138]. In a large-scale survey of 3071 JIA patients, 11 with 4 JSpA had IBD (8 CD, 3 UC); furthermore, there was 1.31 case per 1000 patient-years, higher than the annual incidences of 10 cases per 100000 in pediatric populations of western countries[139]. In another large-scale investigation with 8942 JIA patients, 48 had IBD (22 CD, 13 UC, 13 indeterminate), showing a prevalence of 0.54%, much higher than the reported 0.02% in a Western pediatric population[140]. Furthermore, the occurrences of IBD were identified in 2% to 6% of ERA patients and 0.3% to 0.5% of JPsA patients[133,140,141].

According to the 2019 ACR guidelines for JIA treatment[142], initial therapy with a cDMARD is recommended over NSAID monotherapy, while MTX is suggested over other cDMARDs. Oral CS is only recommended as bridging therapy, with a limited course of less than 3 mo. Furthermore, initial biologic therapy (ADA, ETA, GOL, abatacept, tocilizumab) may be considered for patients with risk factors (seropositivity, articular damage), involvement of high-risk joints (cervical spine, wrist, hip), or high disease activity. For sacroiliitis and enthesopathy, NSAID therapy is recommended, while TNFi is suggested for refractory cases. Notably, UST and SEC have been approved for use in JPsA and ERA/JPsA patients, respectively, as an option for TNFi-resistant patients[143,144]. Although ADA and IFX are both approved in treating pediatric CD and UC, the incidence of IBD in JIA patients was increased in those receiving IFX but not ADA therapy[140]. Furthermore, IFX use is not approved in JIA patients. ADA appears to be a drug of choice for treating patients with JSpA-associated IBD. Nevertheless, there are scarce data regarding ADA use in pediatric patients with joint diseases associated with IBD[145].

Finally, Table 6 lists current FDA-approved indications of biologics and small molecules for seronegative SpA (AS, PsA), JIA and IBD (UC, CD) patients discussed in this review.

CONCLUSION

Seronegative SpA usually starts in the third decade of life with the HLA-B27 genetic marker and clinical features of spinal and peripheral arthritis, dactylitis, enthesitis and EAMs. This group of patients who have negative rheumatoid factor can be classified into AS, PsA, ReA, EnA and JSpA cases. Joint and gut inflammation are intricately linked in SpA and IBD, with shared genetic and immunopathogenic mechanisms. IBD is a common EAM in SpA patients, while EIMs in IBD patients mostly affect the joints. Although individual protocols for managing each disease have been established, the standard therapeutic guidelines of SpA-associated IBD patients remain to be established. NSAIDs are recommended as initial therapy of peripheral and axial SpA, while their use is controversial in IBD due to associated disease flares. cDMARDs are beneficent for peripheral arthritis but ineffective in axial SpA or IBD therapy. Anti-TNF mAbs are effective medications with indicated use in SpA and IBD, being a drug of choice for treating SpA-associated IBD. JAKi, approved in treating SpA and UC, are promising therapeutics in SpA co-existent with UC. A tight collaboration between gastroenterologists and rheumatologists is needed in managing such complex clinical scenarios.

Table 6 Generic names and currently approved indications of biologics and small molecules from the United States Food and Drug Administration for ankylosing spondylitis, psoriatic arthritis, juvenile idiopathic arthritis, and inflammatory bowel disease

Category	AS	PsA	JIA[1]	UC	CD
Biologics/TNFi					
Etanercept	X	X	X		
Infliximab	X	X		X	X
Adalimumab	X	X	X	X	X
Golimumab	X	X	X	X	
Certolizumab pegol	X	X			X
Biologics/IL-17i					
Ixekizumab	X	X			
Secukinumab	X	X	X		
Biologics/IL-12/23i					
Ustekinumab		X	X	X	X
Biologics/IL-23i					
Guselkumab		X			
Risankizumab		X			X
Biologics/IL-1i					
Canakinumab			X		
Biologics/IL-6i					
Tocilizumab			X		
Biologics/anti-integrin mAb					
Natalizumab					X
Vedolizumab				X	X
Biologics/anti-CTLA-4 mAb					
Abatacept		X	X		
Small molecules/JAKi					
Tofacitinib	X	X	X	X	
Upadacitinib	X	X		X	
Small molecules/PDE4i					
Apremilast		X			
Small molecules/S1PR modulator				X	

[1]Tumor necrosis factor inhibitor, abatacept, tocilizumab and tofacitinib for polyarticular juvenile idiopathic arthritis (JIA), canakinumab and tocilizumab for systemic JIA, secukinumab and ustekinumab for juvenile psoriatic arthritis, secukinumab for enthesitis-related arthritis.

AS: Ankylosing spondylitis; CD: Crohn's disease; FDA: United States Food and Drug Administration; IL: Interleukin; JAKi: Janus kinase inhibitor; JIA: Juvenile idiopathic arthritis; PDE4i: Phosphodiesterase 4 inhibitor; PsA: Psoriatic arthritis; S1PR: Sphingosine-1-phosphate receptor; TNFi: Tumor necrosis factor inhibitor; UC: Ulcerative colitis.

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FOOTNOTES

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Country/Territory of origin: Taiwan

ORCID number: Chrong-Reen Wang 0000-0001-9881-7024; Hung-Wen Tsai 0000-0001-9223-2535.

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REVIEW

Review of ferroptosis in colorectal cancer: Friends or foes?

Zheng Wu, Ze-Xuan Fang, Yan-Yu Hou, Bing-Xuan Wu, Yu Deng, Hua-Tao Wu, Jing Liu

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Zheng Wu, Ze-Xuan Fang, Yan-Yu Hou, Jing Liu, Guangdong Provincial Key Laboratory for Diagnosis and Treatment of Breast Cancer, Cancer Hospital of Shantou University Medical College, Shantou 515041, Guangdong Province, China

Bing-Xuan Wu, Yu Deng, Hua-Tao Wu, Department of General Surgery, First Affiliated Hospital of Shantou University Medical College, Shantou 515041, Guangdong Province, China

Corresponding author: Jing Liu, MD, PhD, Academic Research, Associate Professor, Research Scientist, Senior Scientist, Guangdong Provincial Key Laboratory for Diagnosis and Treatment of Breast Cancer, Cancer Hospital of Shantou University Medical College, No. 7 Raoping Road, Shantou 515041, Guangdong Province, China. jliu12@stu.edu.cn

Abstract

Ferroptosis is a newly discovered type of cell-regulated death. It is characterized by the accumulation of iron-dependent lipid peroxidation and can be distinguished from other forms of cell-regulated death by different morphology, biochemistry, and genetics. Recently, studies have shown that ferroptosis is associated with a variety of diseases, including liver, kidney and neurological diseases, as well as cancer. Ferroptosis has been shown to be associated with colorectal epithelial disorders, which can lead to cancerous changes in the gut. However, the potential role of ferroptosis in the occurrence and development of colorectal cancer (CRC) is still controversial. To elucidate the underlying mechanisms of ferroptosis in CRC, this article systematically reviews ferroptosis, and its cellular functions in CRC, for furthering the understanding of the pathogenesis of CRC to aid clinical treatment.

Key Words: Ferroptosis; Colorectal cancer; Cell death; Therapy

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Core Tip: Ferroptosis, a novel type of cell-regulated death, has diverse roles in the occurrence and development of colorectal cancers (CRCs). This article reviews the cellular functions of ferroptosis in CRC, providing potential therapeutic targets and treatment strategies for patients with CRC.

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INTRODUCTION

Regulated cell death, including apoptosis, necroptosis, pyroptosis, ferroptosis, autophagy-dependent cell death, netotic cell death, and other forms, is an important mechanism for regulating the internal environment of the human body, and maintaining tissue function and morphology[1,2]. Ferroptosis, which was formally proposed in 2012, is a unique form of death that depends on the disorder of iron metabolism and accumulation of lipid reactive oxygen species (ROS). It differs from other forms of regulated cell death in terms of morphology, biochemical characteristics, and gene expression[2]. Especially in terms of morphology, ferroptosis involves unique mitochondrial alterations, concerning mitochondrial morphological disorder, membrane potential change, iron overload in the membrane and lipid ROS accumulation, that are different from other death forms[3]. The underlying mechanisms and pathways involved in ferroptosis include glutathione peroxidase/glutathione (GPx/GSH), system Xc^- and p53 regulatory pathways. Usually, the pathways involved in ferroptosis ultimately regulate ROS accumulation through iron accumulation[2,4,5]. At present, the inhibitory effect of ferroptosis on tumor formation and development has been increasingly gaining attention, and its discovery has led to important progress in the diagnosis and treatment of tumors, as well as prognosis.

Colorectal cancer (CRC) is a malignant tumor of the digestive system and is associated with high morbidity and mortality. According to the 2018 GLOBOCAN assessment of global morbidity and mortality, CRC is the third most diagnosed cancer and the second leading cause of cancer-related death globally[6], and is characterized by multiple steps and stages during progression[7]. Currently, treatments for CRC patients include surgery, radiotherapy, chemotherapy, immunotherapy and biological targeted therapy[8]. However, due to the lack of highly specific biomarkers and the complex biological characteristics of CRC, the lack of drugs targeting colorectal stem cells, and chemoresistance or intolerance to current treatment methods continue to hamper treatment[9,10]. Ferroptosis, as a form of regulated cell death independent of other forms of cell death, could provide an effective strategy for CRC treatment. In addition, a large number of recent studies have shown that ferroptosis-related genes can be used to predict the prognosis of patients with CRC, which is of great significance for improving the clinical efficacy of cancer treatment and the survival of patients[11-13].

THE MOLECULAR MECHANISM OF FERROPTOSIS

Discovery of ferroptosis

Erastin, a compound with the ability to kill tumor cells expressing high levels of the *Ras* oncogene, was discovered to induce a novel cell-death form that differed from apoptosis in terms of nuclear morphology, DNA fragmentation and caspase 3 activation[3,14,15]. Although the form of cell-death induced by erastin was not well elucidated at that time, other Ras-selective-lethal compounds (RSLs), such as RSL3 and RSL5, have been shown to trigger the same process, accompanied by increases in ROS levels that could be suppressed by iron chelators[3,16].

Ras protein, encoded by the well-known *RAS* oncogene, binds guanosine 5'-diphosphate (GDP)/guanosine 5'-triphosphate (GTP) and possesses intrinsic GTPase activity[17]. Mutation of Ras is related to the loss of GTPase activity, providing a possible therapeutic strategy of recovering Ras GTPase function in RAS-mutant cancer cells as an effective means to combat cancer[18]. Based on previous findings, Dixon *et al*[2] defined the unique non-apoptotic cell death caused by erastin and RSLs as ferroptosis[2]. Ferroptosis, an iron-dependent form of cell death, is characterized by increases in intracellular ROS, but is distinguished morphologically, biochemically and genetically from other regulated cell death forms, such as apoptosis, necrosis and autophagy, in ways that will be specifically described in the following sections. Since the proposal of the concept of ferroptosis, the mechanism of ferroptosis has become an area of intense research, leading to progress in the study of anti-cancer drugs focused on ROS homeostasis.

The molecular mechanism of ferroptosis

The imbalance between production and degradation of intracellular lipid ROS is the central mechanism of ferroptosis-mediated cell death[2,19]. If the antioxidant capacity of cells is decreased, excessive iron will initiate ferroptosis by producing lethal ROS *via* the Fenton reaction and cause ROS accumulation accordingly[2,5]. In addition, GSH depletion is also important for the induction of ferroptosis and subsequent nicotinamide adenine dinucleotide phosphate (NADPH)-dependent lipid peroxidation[5].

Thus, intracellular ROS accumulation due to iron excess is the key for initiating ferroptosis (Figure 1).

No matter whether using erastin or RSL, the induced ferroptosis is related to iron-dependent accumulation of ROS. In the normal intracellular environment, lipid oxidation and reduction are in a state of dynamic equilibrium. When cellular homeostasis is disrupted, gene expression related to lipid oxidation is up-regulated or that related to lipid reduction is inhibited, causing a high accumulation of intracellular oxidized lipid[20]. However, the sources of ROS are still unclear. Hassannia *et al*[21] pointed out that peroxidation of phospholipids containing polyunsaturated fatty acids (PUFAs) in cell membranes could also lead to ferroptosis[21]. During induction of ferroptosis, PUFAs can form phospholipid hydroperoxides (PLOOHs) through enzymatic or non-enzymatic oxidation reactions. PLOOHs combined with intracellular iron will generate toxic lipid free radicals, such as alkoxy radicals, causing cell damage. Furthermore, these free radicals can extract protons from adjacent PUFAs, initiating a new round of lipid oxidation and delivering further oxidative damage[21,22]. Overall, ROS-mediated cell lipid damage is required for ferroptosis.

Obviously, intracellular iron plays a vital role during the process of ferroptosis, involving the absorption and reduction processes of iron[23]. Iron ingested in food is mainly absorbed, into the blood, as the ferric (Fe^{3+}) form in the duodenum and upper jejunum, and transferred by plasma transferrin into cells, where it is converted to the reduced ferrous (Fe^{2+}) form by metalloreductases in the endoplasmic reticulum (ER). Fe^{2+} is the main form of iron that participates in metabolic processes. Therefore, inhibition of iron absorption and reduction, such as silencing transferrin receptor expression, could inhibit erastin-induced ferroptosis, whereas the elevation of heme catabolism or iron supplementation could restore and accelerate ferroptosis[24-26].

Generally, Fe^{2+} is transported into cells and stored as ferritin to protect cells from iron toxicity[27]. In order to exert biological activity, Fe^{2+} has to be released into the active iron pool in the cytoplasm *via* iron pump solute carrier family 11 member 2/divalent metal transporter 1 (SLC11A2/DMT1), while the extra Fe^{2+} will either be recycled or stored as ferritin[28,29]. Up-regulation of ferritin gene expression restricts iron overload, whereas knockout of the *SLC11A3* gene, which blocks iron transport out of the cells, aggravates erastin-induced ferroptosis in neuroma cells[30-32]. In the case of iron deficiency, ferritin is degraded by autophagy through the ATG5-ATG7-nuclear receptor coactivator 4 (NCOA4) signaling pathway, where NCOA4 binds to and transports ferritin to the lysosome, releasing Fe^{2+} to abnormally increase the labile iron pool. Subsequently, through the Fenton reaction, excess hydroxyl and peroxy radicals can be generated to initiate ferroptosis. Deletion of ATG5, ATG7 or NCOA4 will prevent erastin-induced ferroptosis by limiting ferritin degradation and reducing intracellular ferrous iron levels[33-35].

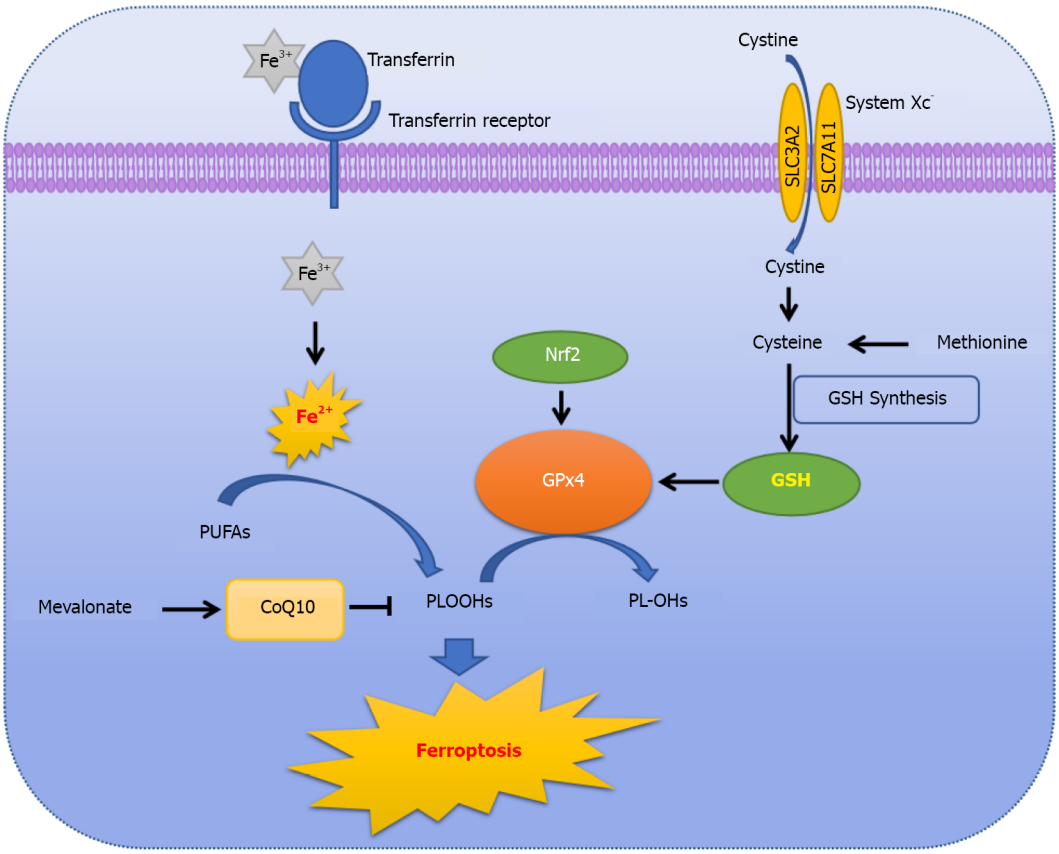
Signaling pathways involved in ferroptosis

The previous findings show that the key link causing ferroptosis involves increased lipid peroxidation and accumulation of ROS. Generally, the ferroptotic upstream pathways ultimately affect the activity of GPx directly or indirectly[4,36,37]. Consequently, GPx family members play an indispensable role in the process of ferroptosis. Among the 8 GPx family members, GPX4, a selenoprotein that inhibits lipid oxidation, has been shown to be the main regulator of ferroptosis[38].

GPX4, a selenoprotein capable of degrading small molecular peroxides and relatively complex lipid peroxides, is also able of reducing cytotoxic lipid hydroperoxides to non-toxic lipid alcohols, preventing the formation and accumulation of lethal ROS[39,40]. Knocking down GPX4 with siRNA results in cell sensitivity to ferroptosis, whereas up-regulating GPX4 induces resistance to ferroptosis[4,36,37]. In fact, RSL3, noted above as an important ferroptotic inducer, can directly suppress the activity of GPX4, thereby inducing ferroptosis[2,41,42]. The selenocysteine active site of GPX4 is covalently bound by RSL3, resulting in reduced cellular antioxidant capacity, increased lipid ROS and initiation of ferroptosis [2,4,43].

Additionally, the biosynthesis of GPX4 occurs through the mevalonate (MVA) pathway *via* interfering with the maturation of selenocysteine tRNAs[43,44]. Selenocysteine is one of the amino acids in the active center of GPX4, and its insertion into GPX4 requires a special selenocysteine tRNA. Isopentenyl pyrophosphate (IPP), a product of the MVA pathway, facilitates the maturation of selenocysteine tRNA by transferring an isopentenyl group to a selenocysteine tRNA precursor through isopentenyltransferase. Importantly, the MVA pathway influences the synthesis of selenocysteine by down-regulating IPP to further disrupt the activity of GPX4, finally causing ferroptosis[45]. Statins, such as cerivastatin, inhibit the MVA pathway and restrict GPX4 biosynthesis[43,44]. Coenzyme Q10 (CoQ10), an endogenous antioxidant produced by the MVA pathway, protects cells from ferroptosis by preventing lipid oxidation[4,37]. Recently, Hadian *et al*[46] implicated ferroptosis-suppressor protein 1 (FSP1) as a novel ferroptosis resistance factor that reduces the expression of CoQ10, leading to the accumulation of lipid peroxides in a process independent of the cysteine/GSH/GPX4 pathway[46].

GSH, a tripeptide antioxidant composed of glutamate, cysteine and glycine[47,48], is an essential cofactor for GPX4 to degrade hydroperoxide[49]. Yant *et al*[50] found that GSH depletion is an indirect way of inactivating GPX4, which further causes a reduction in cellular antioxidant capacity, and increases accumulation of lipid ROS and subsequent ferroptosis[50]. Overall, hindering the synthesis and absorption of GSH or accelerating its degradation provides another means to induce ferroptosis. For example, erastin can block the absorption of GSH by inhibiting system Xc^- to initiate ferroptosis[23].



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Figure 1 The schematic diagram of ferroptosis. PUFAs: Polyunsaturated fatty acids; PLOOHs: Phospholipid hydroperoxides; GSH: Glutathione; Nrf2: Nuclear factor erythroid2-related factor 2; GPx4: Glutathione peroxidase 4.

System Xc^- is a heterodimer composed of solute carrier family 3 member 2 (SLC3A2) and solute carrier family 7 member 11 (SLC7A11), embedded in the cell surface membrane. SLC7A11 is the main functional subunit, which can transport cystine into cells, reduce it to cysteine in the cytoplasm, and incorporate it in the synthesis of GSH[51-54]. Interestingly, inhibiting system Xc^- results in compensatory transcriptional upregulation of SLC7A11 in erastin- and sulfasalazine-induced ferroptosis [54-56]. When system Xc^- is restrained, the absorption of cystine will be hindered, decreasing the synthesis of intracellular GSH, which will interfere with the biological activity of GPX4[22]. Finally, erastin obstructs the absorption of GSH by inhibiting system Xc^-. However, GSH is a necessary cofactor for GPx, so the activity of GPx wanes and eventually results in cell ferroptosis[23]. Nevertheless, ferroptosis inducers that negatively regulate system Xc^- are not effective in killing cells, since the cysteine involved in GSH synthesis can also be synthesized from methionine *via* trans-sulfation. Hayano *et al*[57] showed that inhibition of cysteine tRNA synthetase expression activates the trans-sulfuration pathway, further reducing cellular sensitivity to ferroptosis-inducing agents[57]. In addition, β-mercaptoethanol is able to promote cystine uptake without system Xc^-, thus significantly inhibiting erastin- and glutamate-induced cell death[58].

Another factor involved in ferroptosis is p53, as an important tumor suppressor encoded by *TP53* gene, which is mutated or inactivated in more than half of human cancers[59]. A large number of studies have shown that the tumor suppressing capacity of p53 is mainly derived from its typical functions, such as inducing cell cycle arrest, senescence, or apoptosis[60]. However, p53 also regulates metabolism, metastasis and invasion, and stem cell processes[61]. Recently, atypical functions of p53, such as controlling metabolism and redox status, have also been demonstrated to inhibit tumor development *via* regulating ferroptosis[62,63]. To verify whether p53 could induce ferroptosis, Jiang *et al* [56] showed treatment of p53-mutant non-small cell lung cancer cells with ROS had no significant effect on cell proliferation. However, following re-activation of p53, treatment with ROS dramatically induced 90% cell death, indicating that activation of p53 could dramatically reduce the antioxidant capacity of tumor cells[56]. Under the same conditions, addition of ferrostatin-1, an iron-death inhibitor, reduced the ROS-induced the cell death to 40%, indicating prevention of ROS-induced p53-dependent ferroptosis.

Under circumstances of oxidative stress, p53 can induce ferroptosis by transcriptional inhibition of *SLC7A11*, thereby inhibiting the absorption of cystine and reducing the production of GSH to enhance the sensitivity of cells to ferroptosis[56,64]. It is worth mentioning that acetylation of the p53 DNA-

binding domain plays a key role in the regulation of SLC7A11 expression[56,65]. Notably, mice harboring p53 (3KR), an acetylation-defective p53 due to a lysine-to-arginine mutation, did not form tumors spontaneously, suggesting that p53 (3KR) cells lose their typical functions of inducing apoptosis, senescence, and cell cycle arrest[15], but retain the ability to regulate SLC7A11 expression. This finding highlights the ability of p53 to restrain tumorigenesis by means of inhibiting SLC7A11 expression and triggering ferroptosis[15,56].

Moreover, p53 could promote ferroptosis through regulating its target genes, such as glutaminase 2 (GLS2), prostaglandin endoperoxidase synthetase 2, and spermidine/spermine N1 acetyltransferase 1 (SAT1)[4,56,66,67]. For example, SAT1 enhances the activity of arachidonic acid (AA) and oxidizes PUFAs, thus promoting lipid peroxidation. Knockout of SAT1 will reduce p53-mediated ferroptosis whereas overexpression of SAT1 has the opposite effect[66].

Thus, depending on the p53 mutation status and cellular environment, p53 can promote or inhibit ferroptosis in response to different oxidative stress scenarios[68]. Under high oxidative stress, p53 will promote ferroptosis, while under basal or low ROS stress, it can prevent ferroptosis[69]. On the one hand, activation of the p53-p21 transcriptional pathway enables wild-type p53 to inhibit cysteine deprivation and systemic Xc^- inhibition in cancer cell lines[70], which may help normal cells survive under various metabolic stress conditions. By binding to dipeptidyl peptidase-4 (DPP4) in the nucleus, p53 can prevent the interaction of DPP4 with NADPH oxidase (NOX) in the cytoplasm, and then reduce the accumulation of intracytoplasmic lipid peroxides, thereby inhibiting ferroptosis. This results in p53-WT CRCs being resistant to erastin-induced ferroptosis[71].

Initiation of cystine deprivation-induced ferroptosis requires glutaminolysis[67]. To prevent glutamine hydrolysis and resist ferroptosis, it is possible to restrict the uptake of glutamine by inhibiting the SLC1A5 transporter, inhibit glutamine metabolism to glutamate by mitochondrial GLS2, or deter glutamate synthesis to α-ketoglutarate by aspartate aminotransferase 1[67,72].

FSP1, a novel GSH-independent ferroptosis suppressor, suppresses CoQ10-mediated ferroptosis through an FSP1-CoQ10-NAD(P)H pathway, in a parallel manner to GPX4[73,74]. NADPH, normally used as a biomarker of iron-death inducer sensitivity, is a GSH reductase that maintains reduced GSH [75]. NOX, an enzyme complex that produces superoxide anions and oxidative radicals by consuming NADPH, mediates cellular oxidation to provide an important source of oxidative radicals[2]. Overexpression of NOX causes depletion of intracellular NADPH and increases the level of oxidative free radicals, which significantly raises the sensitivity of cells to ferroptosis. In contrast, NOX inhibitors can down-regulate NOX expression, thereby inhibiting erastin-induced ferroptosis[76].

FERROPTOSIS, A NON-APOPTOTIC CELL DEATH, IS ASSOCIATED WITH MITOCHONDRIAL ALTERATIONS

The characteristics of ferroptosis, compared with other forms of cell death

Ferroptosis, a form of cell death that is different from apoptosis, necrosis and autophagy, depends on the accumulation of lipid ROS, resulting in a redox imbalance. To investigate the differences between ferroptosis and other forms of cell death in morphology, biochemical characteristics, gene expression, and bioenergetics, Dixon *et al*[2] used different inducers to individually induce apoptosis, necrosis and autophagy, and found that, following erastin-induced ferroptosis, cells did not show the morphological characteristics associated with apoptosis (chromatin condensation, plasma membrane blebbing, unique apoptotic bodies), necrosis (cytoplasmic and organelle swelling, cell rupture, cytoskeleton disintegration) or autophagy (formation of a classic closed bilayer structure)[2,14]. Notably, mitochondrial alterations, including small mitochondria, increased membrane density, and mitochondrial outer membrane disruption detected in erastin-treated cells, are unique features that distinguish ferroptosis from other forms of cell death[77-79].

Also, the biochemical characteristics of ferroptosis differ from other forms of cell death. During ferroptosis, Fe^{2+} and ROS accumulate, the mitogen-activated protein kinase (MAPK) system is activated, and the uptake of cystine is reduced, resulting in an inhibitory effect on the Xc^- system. At the same time, this process increases the activity of NOX and promotes the release of mediators, such as AA[1]. Regarding bioenergetics, a large reduction in intracellular ATP is found in H_2O_2-treated, but not in erastin-, STS-, or rapamycin-treated cells[2]. Regarding the characteristics of gene expression in the ferroptosis process, the intracellular Ras/Raf/MAPK and cystine transport pathways, and the activities of acyl-CoA synthetase long-chain family member 4 (ACSL4), NADPH oxidase 1 (NOX1), GPX4, and SLC7A11 were all involved in ferroptosis, which is one of the differences between erastin-induced ferroptosis and other forms of cell death[2].

To investigate the effect of existing cell death inhibitors on erastin-induced ferroptosis, Dixon *et al*[2] used a regulatory assay strategy to test 12 cell death inhibitors for their ability to prevent ferroptosis in cells, and found that compounds confirmed to inhibit apoptosis, necrosis, and autophagy were unable to modulate erastin-induced ferroptosis[2]. In contrast, other compounds, such as the iron chelator deferoxamine (DFO), the antioxidant Trolox, a MEK inhibitor, and the protein synthesis inhibitor cycloheximide, conversely were able to alleviate ferroptosis[3], demonstrating that these compounds are

involved in ROS production and exert a preventive effect on ferroptosis[2].

Unique features of ferroptosis: Mitochondrial alterations

As mentioned above, mitochondrial morphological changes are the most significant feature of ferroptosis compared to other forms of cell death. Dixon *et al*[2] investigated the potential combination of erastin and the voltage-dependent anion channel 2/3 (VDAC2/3) on the mitochondrial membrane by affinity purification, demonstrating that VDAC2 and VDAC3 were necessary but not sufficient for erastin-induced cell death, which also suggests that mitochondria may be involved in the regulation of ferroptosis[3]. Under transmission electron microscopy, it was obvious that the number of mitochondria decreased and bilayer density increased in erastin induced BJeLR cells[2]. Mitochondrial swelling and mitochondrial crests decreased or disappeared in GPX4 ablated cells, and mitochondrial outer membranes rupture in RSL3 exposed Pfa1 cells in a time-dependent manner[37]. These abnormal mitochondrial structural changes are considered to be unique morphological features of ferroptosis[5]. In addition, the latest studies from Dr. Xuejun Jiang's laboratory have shown that cystine starvation-induced ROS accumulation and ferroptosis can be blocked by mitochondrial electron transport chain inhibitors, such as mitochondrial decoupling CCCP with mitochondrial membrane potential disruption [72]. However, in GPX4 knockout-induced ferroptosis, these electron transport chain inhibitors were unable to produce the blocking effect described above[37].

It is well known that iron overload and ROS accumulation are critical processes of ferroptosis in cells, and may be related to their induction of mitochondrial damage[80,81]. Iron overload would lead to mitochondrial morphological abnormalities, limit mitochondrial oxidative phosphorylation and antioxidant reactions, and impair mitochondrial function. Mitochondrial DNA (mtDNA) double-strand breaks, reduced mtDNA transcription, and decreased expression of respiratory chain subunits encoded by the mitochondrial genome have been observed in iron-overloaded mitochondria[82-85], whereas preserving mitochondrial structure and function protects cells from iron toxicity[86]. In addition, Carsten Culmsee and colleagues found a sharp increase in mitochondrial ROS in erastin-[87] and RSL3-treated[88] HT-22 and MEF cells, but not in erastin treated HT-1080 cells. Thus, the researchers speculated that the difference could be due to the use of different cells or different exposure times. The mitochondrial targeting ROS scavenger MitoQ (mito-quinone) prevents neuronal cells from undergoing RSL3-induced ferroptosis[88]. Other studies have indicated that lipid ROS accumulates in the mitochondria rather than the cytoplasm[89], while some reports suggest that ferroptosis is caused by lipid peroxidation outside the mitochondria[37].

In general, according to existing studies, the structural integrity of mitochondria becomes damaged, membrane potential is altered, and abnormal iron metabolism and lipid peroxidation have varying degrees of influence on mitochondrial function. However, the alterations in mitochondrial structure and function in ferroptosis still requires further exploration and verification.

The surefire way to ferroptosis: Lipid peroxidation

Based on lipomics analysis, polyphosphorylated phosphatidylethanolamines (PEs) have been found to be key components in the induction of ferroptosis[90,91]. ACSL4, a key enzyme regulating lipid composition, catalyzes the addition of coenzyme A to AA and adrenic acid (AdA) to form PUFA coenzyme derivatives AA-CoA and AdA-CoA through an ER-associated oxygenation center. Subsequently, AA-CoA and AdA-CoA are esterified to AA-PE and AdA-PE by lysophosphatidylcholine acyltransferase 3 (LPCAT3) to take part in the synthesis of membrane phospholipids with negative charge[91-94]. In this situation, downregulation of ACSL4 for better conversion of AA to acylated AA, or inactivation of LPCAT3 to catalyze the insertion of acylated AA into membrane phospholipids, are also effective approaches to induce resistance to ferroptosis[90-92,95].

Additionally, free PUFAs such as AA-PE and AdA-PE will be selected as the preferred substrates for lipoxygenases (LOXs), lipid peroxidizing enzymes that catalyze the peroxidation of unsaturated fatty acids[96]. Knocking out LOX expression or treatment of cells with both tocotrienols and tocopherols have become an effective means to reduce erastin-induced ferroptosis[97,98]. With such treatment, LOX binds to phosphatidylethanolamine-binding protein 1 (PEBP1) to form a 15-LOX/PEBP1 complex that oxidizes AA-PE and AdA-PE to lipid hydroperoxides, thereby co-regulating the oxidation and reduction of esterified fatty acids with recombinant GPX4[99,100].

Recently, nuclear factor erythroid 2-related factor 2 (Nrf2), a well-known transcription factor, was found to be involved in the antioxidant process by inducing many antioxidant enzymes with antioxidant response elements in their promoters, such as GPX4 and GSH reductase, and its activity is strictly controlled by Kelch-like ECH-associated protein 1 (KEAP1)[101]. Under normal conditions, the binding of Nrf2 to KEAP1 causes the degradation and inactivation of Nrf2. However, when in a state of oxidative stress or with a large number of electrophiles, Nrf2 is released from the KEAP1 binding site and rapidly enters the nucleus to balance oxidative stress by activating transcriptional pathways and maintaining cellular redox homeostasis, ultimately inhibiting cellular oxidation and ferroptosis[74,102].

FERROPTOSIS PARTICIPATES IN TUMOR OCCURRENCE AND PROGRESSION

Ferroptosis and the tumor microenvironment

Ferroptosis can either inhibit or enhance tumorigenesis and development, with enhancement depending on the release of damage-associated molecular patterns in the tumor microenvironment (TME) and the inhibition of anti-tumor immune mechanisms, and inhibition depending on the activation of immune responses triggered by ferroptosis injury[103]. Therefore, understanding the interaction between ferroptosis and TME could provide new and effective anti-cancer strategies[104].

The TME is a complex environment within the tumor and enables tumor cells to survive and develop, serving as the 'soil' for non-cancerous cells (including stromal cells, immune cells, adipocytes, and endothelial cells) and extracellular matrix[105]. Theoretically, changes in tumor cytogenetics and epigenetics could enhance the ability of cancer cells to evade immune surveillance through various metabolic and biochemical mechanisms, ultimately promoting tumor initiation, progression, and metastasis[106]. Recently, Dai *et al*[107] found that ferroptosis caused by autophagic degradation releases cancer cells into TME and drives tumor-associated macrophage (TAM) polarization[107]. Yet ferroptosis inducers, such as erastin, RSL3 and sulfasalazine, have the capability to induce ferroptosis in cancer cells through different pathways, thus exerting anti-cancer effects, indicating the diverse role of ferroptosis in the process of cancer.

The regulatory effects of ferroptosis in malignancies

Erastin-induced cell death can be inhibited by antioxidants and iron chelators, suggesting that erastin-triggers cell death *via* ferroptosis related to the accumulation of ROS and iron[2,3]. The reduced GSH levels caused by erastin results from direct inhibition of the Xc^- system, activating the ER stress response, and attenuating the antioxidant effect of GPX4/GSH, thereby accelerating the accumulation of ROS in the cytoplasm[42]. Sulfasalazine, another inducer of ferroptosis, has the same mechanism and function as erastin[108]. Thus, treatment with sulfasalazine also induces ferroptosis in cancer cells[2,42]. Activation of the RAF/MEK/ERK signaling pathway also appears to be an important factor in erastin-treated cells with high Ras expression[3]. As mentioned before, changes in mitochondrial structure and morphology are detected in cells following treatment with erastin, which binds to the mitochondrial VDAC[3].

Other inducers of ferroptosis, such as RSL3 and ferroptosis-inducing agents (FINs), are also associated with ROS accumulation. RSL3, a direct inhibitor of GPX4, can inactivate GPX4 through direct binding, resulting in the accumulation of intracellular lipid peroxides and ferroptosis[4]. FINs, with the ability to generate ROS, are classified into two types according to their mechanism of action[109,110]. Class I FINs share the same mechanism as erastin and sulfasalazine, inducing GSH depletion[111,112]. Class II FINs, similar to RSL3, directly inhibit the activity of GPX4 without depleting GSH[4]. Yang *et al*[4] showed that GPX4 overexpression and knockdown modulated the lethality of 12 ferroptosis inducers, but not of 11 compounds that induced cell death by other mechanisms[4].

Nevertheless, erastin- and RSL3-induced ferroptosis could be inhibited by ferrostatin, a lipophilic iron chelator[2]. Ferrostatin can cross the cell membrane and chelate free intracellular iron, or act directly on enzymes containing iron, to prevent the formation of iron-catalyzed lipid free radicals and inhibit the degradation of PUFAs. Lipoxygenase can be directly inactivated by iron chelators and thus most likely mediates iron-dependent lipid ROS formation[113]. Unlike lipophilic iron chelators, DFO is a non-membrane-permeable chelator that can accumulate on cell lysosomes, following endocytosis, and interact with iron in lysosomes to prevent the generation of lipid ROS[113-115]. Liproxstatin-1, a clinical drug, acts by preventing the accumulation of ROS and cell death in $GPX4^{-/-}$ cells[37]. More importantly, liproxstatin-1 is able to inhibit FIN-induced ferroptosis *in vitro*[37].

MECHANISMS OF FERROPTOSIS IN CRC

Signaling pathways of ferroptosis involved in CRC

GPX4 is a key factor in the regulation of ferroptosis. Molecules that inhibit GPX4 activity, either directly or indirectly, are involved in ferroptosis. RSL3, a confirmed ferroptosis-inducer, has anti-cancer effects in CRC that can be enhanced by aspirin through suppressing mechanistic target of rapamycin (mTOR)/sterol regulatory element-binding protein 1 (SREBP-1)/stearoyl-CoA desaturase-1 (SCD1)-mediated lipogenesis in PIK3CA-mutant CRC[116]. Not surprisingly, genetic ablation of SREBP-1 or SCD1 expression enhances CRC cell sensitivity to RSL3-induced ferroptosis, supporting the molecular mechanism of aspirin on RSL3-induced cytotoxicity[116].

In addition, reducing the synthesis of intracellular GSH by inhibiting SLC7A11, which is also the target of erastin and sulfasalazine, is also an effective way to induce ferroptosis in CRC[117]. 2-Imino-6-methoxy-2H-chromene-3-carbothioamide, a benzopyran derivative, has also been reported to have anti-cancer activity and was first discovered to exert ferroptotic anti-CRC activity through down-regulating SLC7A11 expression in the AMP-activated protein kinase/mTOR/p70S6K pathway in CRC[118].

Importantly, cancer stem cell (CSC)-regulated phenotypic plasticity and protection of metastasized cancer cells from lipid peroxidation and ferroptosis are related to the increased expression of SLC7A11 [119]. In CRC, a remarkably low level of ROS is found in colorectal CSCs high in cysteine, GSH and SLC7A11 compared with CRC cells, while targeting SLC7A11 could induce ferroptosis through specifically suppressing the progression of colorectal CSC. Erastin exerts a dramatically strong cytotoxic effect on colorectal CSCs *in vitro* and *in vivo*[120]. By comparing the stemness of CRC cells and drug-resistant cells, it was found that the higher the stemness of CRC tumors, the more obvious the anti-ferroptosis characteristics. Correspondingly, the higher the stemness of CRC, the higher the expression of SLC7A11, suggesting that SLC7A11 is a potential target for colorectal CSC resistance to ferroptosis [120,121]. Knockdown of SLC7A11 expression in CSCs induced down-regulation of ALDH1, and tumor sphere size, as well as decreased cysteine and GSH and increased ROS levels, indicating decreased tumor stemness and increased ferroptotic characteristics. Similar results were obtained with erastin treatment, suggesting that erastin can effectively induce ferroptosis in drug-resistant CRC cells, thus achieving a therapeutic effect by targeting drug-resistant CSCs[120,121], providing a potential solution for drug resistance in CRC.

Doll *et al*[91] performed a genome-wide CRISPR-based genetic screen and microarray analysis of ferroptosis-resistant cell lines, and identified ACSL4 as an essential component for the synthesis of PE and execution of ferroptosis[91]. Another study analyzed the signaling pathway and miRNA profile of Kras mutant human CRC cells, and found that ACSL4 expression is high in CRC cells. Bromelain, a plant extract derived from pineapple, stimulated the expression of ACSL4, induced the cells to undergo ferroptosis and inhibited tumor progression[122], suggesting that the Kras gene may be an upstream regulator of ferroptosis.

TP53, an important tumor suppressor, plays a tumor suppressor role through transcriptional or non-transcriptional mechanisms in cancer cells[71]. In CRC cells, wild-type p53 restrains ferroptosis by blocking DPP4 activity, while deletion of wild-type p53 increases the anti-cancer activity of erastin in tumor-bearing mice[71]. In the nucleus, wild-type p53 binds to DPP4, preventing the translocation of DPP4 from the nucleus to the cytoplasm and the formation of the DPP4-NOX1 complex, responsible for preventing lipid peroxidation and ferroptosis, thereby restoring erastin-induced sensitivity in CRC cells [64,71]. On the contrary, p53 can also stimulate the expression of SLC7A11 in CRC, thereby protecting CRC cells from ferroptosis[71]. Therefore, it is desirable to modulate p53 to achieve efficacy in future CRC treatments.

Although ferroptosis is markedly different from other forms of cell death, there is a link between them in CRC. Hong *et al*[123] treated cancer cells with a combination of an apoptotic agent [tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)] and ferroptotic agents (erastin or artesunate) and found molecular crosstalk between ferroptosis and apoptosis[123]. The combined treatment remarkably promoted TRAIL-induced apoptosis due to the expression of ER stress-induced p53-independent up-regulation of apoptosis regulator PUMA. Further experiments found that the ER stress-response mediated by death receptor 5, one of the TRAIL receptors, also plays a significant role in the combined synergistic cytotoxic effect on multiple cell lines[124]. On the other hand, iron autophagy promotes ferroptosis in various types of cancer cells through regulating NCOA4, and inhibition of ferritin degradation inhibits the ferroptosis of these cells[31,35]. Interestingly, knockdown of NCOA4 did not change the ferroptosis of CRC cells[125], which could be explained by cell line differences or NCOA4 functional compensation, but further studies are needed.

Recent progress in CRC ferroptosis

In addition to the classical pathways mentioned above, mechanistic studies on ferroptosis in CRC are also increasing. A recent study revealed TP53-induced glycolysis and apoptosis regulator (TIGAR) to be a potential inhibitor of ferroptosis during CRC development[126]. TIGAR is highly expressed in CRC cell lines, and knockdown of TIGAR unexpectedly increases erastin-induced growth inhibition and death, indicating that low levels of TIGAR increase the sensitivity of CRC cells to erastin-induced ferroptosis and that TIGAR is a potential negative regulator of ferroptosis. Increased levels of lipid peroxidation and malondialdehyde are associated with knockdown of TIGAR in CRC, without obvious changes of iron level, suggesting TIGAR is a potential target for iron-death-based therapy for CRC through regulating ROS[126]. Similarly, cytoglobin (CYGB), a regulator of ROS, has been shown to be an inhibitor of ferroptosis *via* the p53-YAP1 pathway. In the same study, CYGB suppression, first shown in CRC, promoted ROS production and increased the sensitivity of cancer cells to RSL3- and erastin-induced ferroptosis, thus inhibiting the growth of CRC cells in a YAP1-dependent manner[126].

As GPX4 also plays crucial roles in ferroptosis, factors regulating GPX4 are predicted to be involved in the regulation of ferroptosis. Lipocalin-2 (LCN2), a protein siderophore that regulates iron homeostasis, is upregulated in several types of tumors, including CRC. Overexpression of LCN2 reduces the level of ferroptosis through reducing intracellular iron levels and stimulating the expression of GPX4 and system Xc$^-$[127]. Serine and arginine rich splicing factor 9 was identified as a key factor promoting GPX4 expression and correspondingly decreased lipid peroxide damage, thereby driving CRC tumorigenesis, and thus providing another target for enhancing the sensitivity of CRC to erastin[128]. In addition, miR-15A-3p was found to positively regulate ferroptosis by directly targeting and suppressing GPX4 in CRC[129].

GTP cyclohydrolase 1 (GCH1), a rate-limiting enzyme in the synthesis of the free radical trapping antioxidant tetrahydrobiopterin (BH4) was found to suppress ferroptosis in a GPX4-independent manner[130]. Blocking GCH1/BH4 promoted erastin-induced but not RSL3-induced ferroptosis, suggesting that GCH1 inhibitors combined with erastin provide a novel treatment strategy for CRC [130]. Interestingly, autophagy inhibitors could reverse erastin resistance in GCH1-knockdown cells, suggesting that GCH1/BH4 may act through ferritin phagocytosis[130]. Another novel inducer of ferroptosis, talaroconvolutin A was found to strongly induce ferroptosis in a dose- and time-dependent manner, but not apoptosis. Surprisingly, talaroconvolutin A was far more effective in inhibiting CRC by ferroptosis than erastin, and thus has become a potential treatment option for inducing ferroptosis in CRC[131].

CLINICAL IMPLICATIONS OF FERROPTOSIS INDUCTION IN CANCER TREATMENT

Cancer remains one of the most threatening diseases to human health. Although traditional treatments, such as medication, surgery, radiation and chemotherapy, and comprehensive treatment, as well as immune therapy and targeted therapy, have been applied in the clinic, but the complexity of cancer pathogenesis, drug resistance, and patient intolerance have severely limited the efficacy of these approaches[132]. Therefore, further investigation is needed to explore the molecular changes and mechanisms involved in tumorigenesis and prognosis. Ferroptosis, a novel form of death, could play an indispensable role in inhibiting tumor growth and may therefore become an emerging strategy for anti-cancer therapy.

Reversing chemotherapeutic drug resistance

Chemotherapy has remained a necessary means to treat cancer, but drug resistance also remains one of the reasons for the poor prognosis of patients with malignancies. According to the molecular mechanism of ferroptosis, the pathways that reduce chemotherapeutic drug resistance are mainly involved in the lipid metabolism, iron metabolism and classical GPX4 pathways. The resistance of chemotherapeutic drugs in CRC also involves these processes.

In the process of lipid metabolism, ACSL4 is involved in the lipid oxidation pathway through the conversion of AA and AdA in PUFAs into coenzyme derivatives, and then producing oxidized lipid molecules[91]. Wu *et al*[133] reported that inhibiting ADP ribosylation factor 6 (ARF6), functions downstream of the Kras/ERK signaling pathway, and can activate ACSL4 and endow cancer cells with sensitivity to oxidative stress, especially RSL3-induced lipid peroxidation. ARF6 has a profound effect on the development of pancreatic cancer. Abrogation of ARF6 promotes RSL3-induced ferroptosis and alleviates gemcitabine resistance[133]. Another key enzyme in lipid metabolism, LOX, can directly oxidize PUFAs and mediate ferroptosis in a non-enzymatic manner[97]. Wu *et al*[134] demonstrated that arachidonate lipoxygenase 15 (ALOX15) is closely related to the inhibition of ferroptosis in gastric cancer. Decreasing miRNA-522 and increasing ALOX15, to induce ferroptosis, has become a novel treatment strategy to reverse drug resistance in gastric cancer, especially resistance to cisplatin/paclitaxel[134].

In iron metabolic pathways, dihydroartemisinin (DHA), a safe and promising therapeutic agent that preferentially induces ferroptosis of cancer cells, was found to intensively enhance the cytotoxicity of cisplatin through impairing mitochondrial homeostasis and increasing mitochondrial-derived ROS, as well as promote ferroptosis with catastrophic accumulation of free iron and unrestricted lipid peroxidation. Depleting the free iron reservoir prevents death and triggers tolerance to DHA/cisplatin-induced ferroptosis, whereas supplementation of iron accelerates ferroptotic cell death[135]. Blocking lysosomal iron translocation out of lysosomes can be caused by the inhibition of DMT1 in CSC, resulting in iron accumulation in lysosomes, production of ROS and cell death in the form of ferroptosis[136].

Chen *et al*[137] discovered that androgen receptors could induce tumor cell drug resistance during the treatment of glioblastoma with temozolomide. Curcumin analogues reverse temozolomide resistance through ubiquitinating androgen receptors, which can be achieved by inhibition of GPX4 followed by induction of ferroptosis[137]. The reduction of oxaliplatin resistance in CRC occurs through a similar mechanism. CRCs induce ferroptosis by disrupting the KIF20A/NUAK1/PP1β/GPX4 pathway, in which high expression of KIF20A has been shown to be associated with oxaliplatin resistance[138]. In addition to the direct inhibition of GPX4 activity, blocking the synthesis of GSH also triggers ferroptosis indirectly. It is reported that ent-kaurane diterpenoids overcome cisplatin resistance by targeting peroxiredoxin I/II and consuming GSH to induce ferroptosis[139]. In head and neck cancer, cisplatin resistance can also be overcome by inhibiting system Xc^-[140], and in gastric cancer, inhibition of the Nrf2/Keap1/system Xc^- signaling pathway can induce the same effect[141].

Reversing targeted-therapy resistance

However, chemotherapy as a major means of cancer treatment has the undesirable side-effect of killing normal cells. In contrast, targeted therapy has gradually become an effective treatment. About half of patients with metastatic CRC have *RAS* mutations, which greatly limit the efficacy of cetuximab, an

anti-epidermal growth factor receptor antibody. A natural product β-elemene, isolated from the Chinese herb turmeric, in combination with cetuximab, confers high cytotoxicity toward metastatic CRC cells with Kras mutations, and works by inducing ferroptosis and inhibiting epithelial-mesenchymal transition[9]. Olaparib, a well-known inhibitor of poly (ADP-ribose) polymerase, promotes ferroptosis by inhibiting SLC7A11-mediated GSH synthesis. A synergistic effect with FINs can sensitize BRCA-activated ovarian cancer cells and xenograft cells[142]. Triple-negative breast cancer (TNBC) cells are resistant to clinical doses of gefitinib. Inhibition of GPX4 and induction of ferroptosis can enhance the sensitivity of TNBC to gefitinib[143]. Sorafenib is the first approved systemic medicine for advanced hepatocellular carcinoma, but acquired resistance limits its usefulness in the clinic. Inhibition of metallothionein-1g expression can enhance the anti-cancer activity of sorafenib by inducing ferroptosis *in vitro* and *in vivo*[144]. Artesunate, a drug derived from traditional Chinese medicine, inhibits the growth of sunitinib-resistant renal cell carcinoma by cell cycle arrest and induction of ferroptosis[145]. Similar to erastin, GSH depletion accompanied by GPx inactivation is the underlying mechanism of cisplatin, and cisplatin combined with erastin has enhanced anti-tumor activity compared to cisplatin alone[10]. The combination of erastin and cisplatin may be a useful strategy to improve the efficacy of cisplatin for the reason that the mechanisms used by these two compounds are different[10,146].

Reversing immunotherapy resistance

Over the past decade, immunotherapy with immune checkpoint inhibitors has shown promising efficacy in various malignancies. Even so, there has been some resistance to its use. Based on research advances, it has been proposed that stimulating the adaptive immune system by promoting immunogenic cell death may change the immune cold state into a checkpoint blockade response state, and ferroptosis happens to be immunogenic[147,148]. Therefore, induction of ferroptosis in cancer cells may induce vaccine-like effects and stimulate anti-tumor immunity, thereby overcoming immunotherapy resistance[149-151]. On the other hand, immunosuppressive cells in the TME also contribute to immunotherapy resistance, such as regulatory T cells (Tregs) and TAMs[152]. These findings suggest that induction of ferroptosis in Tregs by GPX4 inhibition may reverse immunotherapy resistance[153]. Moreover, Jiang *et al*[154] found that reprogramming of TAMs, due to in-tumor cell ferroptosis, resensitizes the tumor cells to immunotherapy[154].

CLINICAL PROGNOSTIC MODEL FOR FERROPTOSIS IN CRC

In recent years, numerous studies have focused on genetic screening for colon cancer and the establishment of polygenic prognostic models associated with ferroptosis. Owing to the lack of reliable and accurate biomarkers, the diagnosis, treatment and prognosis of colon cancer are faced with great challenges. Therefore, the establishment of a sound prognostic model and the mining of key biomarkers are effective ways to accurately predict the prognosis of CRC patients. In addition to their anti-cancer potential, ferroptosis-related genes also play an important role in the construction of prognostic models. Recent studies have shown that a new ferroptosis-related 10-gene prognostic model effectively predicts the prognosis and overall survival of patients with CRC, providing a reference value for targeted therapy and immunotherapy[11]. Xiang *et al*[12] established a prediction model based on regression analysis of CRC differentiation-related genes (CDRGs), and found that down-regulation of CDRGs was closely related to ferroptosis and immune metabolism, thus showing that molecular subtypes based on cell differentiation can successfully predict the prognosis of CRC patients undergoing chemotherapy and immunotherapy[12]. Moreover, a prognostic model based on EMT and ferroptosis-related genes predicted the ability of colorectal adenocarcinoma to invade and metastasize, where four genes involved in ferroptosis were potential prognostic biomarkers, thus providing important guidance for the individualized treatment and clinical decision-making of CRC[13]. Additionally, ferroptosis-related lncRNA signatures have proved to be promising biomarkers. Wu *et al*[155] constructed a robust prognostic model with only 4 ferroptosis-related lncRNA signatures, and the signature-based risk score showed a stronger ability to predict survival than traditional clinicopathological features, contributing to the prediction of clinical outcomes and treatment responses in patients with colon cancer[155] (Table 1). In future studies, the construction of CRC prognostic models and the discovery of potential biomarkers are expected to enhance and improve the survival and prognosis of CRC patients.

CONCLUSION

In summary, ferroptosis mainly involves the accumulation of intracellular lipid ROS resulting from a disorder of iron metabolism[2]. Its unique form of death provides potential targets for the treatment of tumors. Since ferroptosis exerts different effects through different mechanisms in different tumor types, this article focuses on CRC to elaborate the molecular mechanisms and pathways of ferroptosis.

Table 1 Prognostic model of ferroptosis-related genes in colorectal cancer patients

Model	Related genes	Ref.
10-Gene prognostic model	*TFAP2C, SLC39A8, NOS2, HAMP, GDF15, FDFT1, CDKN2A, ALOX12, AKR1C1, ATP6V1G2*	[11]
Prediction model based on CDRG[1] regression analysis	*ACAA2, SRI, UGT2A3, KPNA2, MRPL37*	[12]
Prognostic model based on EMT[1] and FRGs[1]	*MMP7, YAP1, PCOLCE, HOXC11*	[13]
Prognostic model of 4-FRL[1] signatures	*AP003555.1, AC104819.3, LINC02381, AC005841.1*	[155]

[1]CDRG: Colorectal cancer differentiation-related gene.
EMT: Epithelial-mesenchymal transition; FRG: Ferroptosis-related gene; FRL: Ferroptosis-related lncRNA.

Many studies have proposed possible pathways of ferroptosis in CRC, but the specific mechanisms involved in the occurrence, development and metastasis of CRC remains unclear. In the classical pathway, GPX4 can be used as a target for tumor therapy, but the inhibition of GPX4 may cause side effects due to its protective role against β-amyloid toxicity in neurons[156,157]. Additionally, p53 has contradictory effects on ferroptosis, but the mechanism in CRC is unique. Future studies can explore how to achieve the switch between "brake" and "accelerator" in the regulation of ferroptosis[71]. Generally, the role of ferroptosis in disease may be to promote[158,159] or inhibit, but the induction of ferroptosis, undoubtedly, has an inhibitory effect on the occurrence, development and metastasis of CRC. In addition to the classical mechanism, other potential regulatory pathways need to be discovered. As mentioned above, these regulated types of cell death may share common pathways and key regulators, which could provide new directions for combined therapeutic interventions. Furthermore, the occurrence of ferroptosis is cell-type dependent, so cancer treatment based on ferroptosis will not necessarily be suitable for all cancer types, or even for different clinical stages of the same type. Although these treatments can be expected to be affected by the development of tumor cell drug resistance, with the gradual development of research, reducing drug resistance by inducing ferroptosis is gradually becoming a reality[10], and the construction of prognostic disease models by screening ferroptosis-related genes has become a focus of clinical research[13]. However, translating the multiple basic research discoveries in ferroptosis to clinical treatment will be another difficult problem we will soon face.

FOOTNOTES

Author contributions: Liu J and Wu HT designed the research study; Wu Z, Fang ZX, Hou YY, Wu BX and Deng Y performed the research; Wu Z, Fang ZX, Hou YY, Wu BX and Deng Y analyzed the data and wrote the manuscript; Liu J revised the manuscript critically; All authors have read and approve the final manuscript.

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Country/Territory of origin: China

ORCID number: Zheng Wu 0000-0002-1393-7586; Ze-Xuan Fang 0000-0002-6100-9012; Yan-Yu Hou 0000-0002-4249-8770; Bing-Xuan Wu 0000-0002-1212-7936; Yu Deng 0000-0002-0950-6169; Hua-Tao Wu 0000-0002-1640-6094; Jing Liu 0000-0002-7483-4572.

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MINIREVIEWS

Clinical implications of COVID-19 in patients with metabolic-associated fatty liver disease

Mohammad Sadiq Jeeyavudeen, Rahul Chaudhari, Joseph M Pappachan, Sherouk Fouda

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Mohammad Sadiq Jeeyavudeen, Department of Endocrinology and Metabolism, University Hospitals of Edinburgh, Edinburgh EH4 2XU, United Kingdom

Rahul Chaudhari, Department of Gastroenterology, Hepatology and Nutrition, Virginia Commonwealth University, Richmond, VA 23298, United States

Joseph M Pappachan, Department of Endocrinology and Metabolism, Lancashire Teaching Hospitals NHS Trust, Preston PR2 9HT, United Kingdom

Joseph M Pappachan, Faculty of Science, Manchester Metropolitan University, Manchester M15 6BH, United Kingdom

Joseph M Pappachan, Faculty of Biology, Medicine and Health, The University of Manchester, Manchester M13 9PL, United Kingdom

Sherouk Fouda, School of Health and Biomedical Sciences, RMIT University, Melbourne VIC, Australia

Corresponding author: Joseph M Pappachan, FRCP, MD, Consultant, Professor, Senior Editor, Senior Researcher, Department of Endocrinology and Metabolism, Lancashire Teaching Hospitals NHS Trust, Sharoe Green Lane, Preston PR2 9HT, United Kingdom.
drpappachan@yahoo.co.in

Abstract

People across the world are affected by the "coronavirus disease 2019 (COVID-19)", brought on by the "SARS-CoV type-2 coronavirus". Due to its high incidence in individuals with diabetes, metabolic syndrome, and metabolic-associated fatty liver disease (MAFLD), COVID-19 has gained much attention. The metabolic syndrome's hepatic manifestation, MAFLD, carries a significant risk of type-2-diabetes. The link between the above two conditions has also drawn increasing consideration since MAFLD is intricately linked to the obesity epidemic. Independent of the metabolic syndrome, MAFLD may impact the severity of the viral infections, including COVID-19 or may even be a risk factor. An important question is whether the present COVID-19 pandemic has been fueled by the obesity and MAFLD epidemics. Many liver markers are seen elevated in COVID-19. MAFLD patients with associated comorbid conditions like obesity, cardiovascular disease, renal disease, malignancy, hypertension, and old age are prone to develop severe disease. There is an urgent need for more studies to determine the link between the two conditions and whether it might account for racial

differences in the mortality and morbidity rates linked to COVID-19. The role of innate and adaptive immunity alterations in MAFLD patients may influence the severity of COVID-19. This review investigates the implications of COVID-19 on liver injury and disease severity and vice-versa. We also addressed the severity of COVID-19 in patients with prior MAFLD and its potential implications and therapeutic administration in the clinical setting.

Key Words: Metabolic-associated fatty liver disease; COVID-19; Metabolic syndrome; Non-alcoholic steatohepatitis; Angiotensin converting enzyme 2

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Core Tip: The severity of coronavirus disease 2019 (COVID-19) symptoms and sequelae positively correlate with high rates of hepatic decompensation and elevated transaminases in patients with chronic liver disease and cirrhosis. Implicated mechanisms linking cirrhosis with severe COVID-19 symptoms include cirrhosis-related immune dysregulation, systemic inflammation, coagulopathy, and metabolic derangements. Metabolic-associated fatty liver disease (MAFLD) is characterized as the hepatic manifestation of the metabolic syndrome and therefore is highly associated with other comorbidities such as obesity, diabetes, and hyperlipidemia. Those comorbidities are also risk factors for severe COVID-19. The hepatic distribution of the angiotensin-converting enzyme 2 receptor, the main viral entry receptor for SARS-CoV-2, may determine the severity of hepatic involvement. In addition, moderate hepatic dysfunction could alter the severity of COVID-19, as well as the safety profile, and the therapeutic efficacy of antiviral drugs metabolized in the liver. Therefore, it is of high clinical priority to enhance our understanding of COVID-19 infection-associated liver injury in MAFLD patients to treat both of these conditions effectively.

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INTRODUCTION

A substantial hazard to public health has suddenly emerged from the "severe acute respiratory syndrome (SARS)" global pandemic caused by the coronavirus SARS-CoV-2 "[coronavirus disease 2019 (COVID-19), *Sarbecovirus subgenus*, *Betacoronavirus genus*, *Coronaviridae family*]"[1-5]. Up until the September 2, 2022, the total infected cases were 607013841; total deaths were 6508326 and total vaccinated were 12185442365[2,6]. The most common and important clinical manifestation of COVID-19, alternating from moderate respiratory symptoms to severe pneumonia, is respiratory involvement, even though many people still show no symptoms. The severe Corona virus infection, however, is a systemic illness that can cause myocardial injury, heart failure, vascular inflammation, myocarditis, cardiac arrhythmias, hypoxic encephalopathy, multi-organ failure, and eventually death[1-5,7-11].

Though severe liver damage is rare, the liver remains a potential target for Coronavirus. This infection poses a novel challenge for hepatologists because it may harm the liver by direct (viral translocation from the gastrointestinal tract to the liver) or indirect pathways (systemic inflammation, hepatic ischemia and hypoxia, effects on pre-existing liver illnesses, and drug-related liver injury)[12-17].

Notably, nonalcoholic fatty liver disease (NAFLD), a chronic dysmetabolic pandemic with a prevalence rate of > 30% in the global population, has become the most widespread liver disease in the world. Furthermore, NAFLD is a "fellow traveler" with a number of risk factors, metabolic syndrome, and diseases rather than a stand-alone disorder. Along with this viewpoint, the term "metabolic-associated fatty liver disease" (also known as "MAFLD") has recently given the acronym NAFLD a second look[18,19]. Therefore, NAFLD/MAFLD may impact how COVID-19-infected "patients" fare. Additionally, in situations of chronic injury, the liver itself is more vulnerable to medicines.

In this setting, individuals with NAFLD/MAFLD and COVID-19 infections exhibit inflammatory response pathways, particularly those involving cytokines that may aggravate the clinical result by causing an increase in liver inflammation or by serving as a marker of metabolic risk factors. A precise understanding of the behavior of the virus and the risk factors contributing to the initiation and progression of COVID-19 will be crucial in the near future to predict virus-related events around the globe due to the pandemic characteristics and high mortality rate of SARS-CoV-2 infection.

According to Wang *et al*[20], analysis of COVID-19-infected patients revealed independent risk factors for hypertension, diabetes, chronic obstructive pulmonary disease, cardiovascular disease, and cerebrovascular disease [odds ratio (OR): 2.29-5.97]. A recent study on COVID-19-infected individuals who were hospitalized in New York reported that 48.7% of the patients had a BMI > 40 kg/m^2, suggesting that BMI is one of the strongest predictors of hospitalization (OR: 6.2), only being surpassed by ages ≥ 75 years (OR: 66.8) and age 65-74 years (OR: 10.9)[21]. Finally, MAFLD was found to be independently linked with COVID-19 progression in a study of 202 consecutive individuals with confirmed COVID-19[22]. Acute COVID-19 epidemic and chronic MAFLD, which is a member of a larger group of metabolic illnesses, are the two pandemic conditions that are the subject of this article's discussion. The underlying MAFLD may contribute to more severe hepatic and metabolic consequences during COVID-19 infection and may develop into another prognostic indicator of the viral illness[21-26].

COVID-19 AND MAFLD

In contrast to the hepatocytes which are the predominant liver cells and of which only 3% express angiotensin I converting enzyme 2 (ACE2) receptors, about 60% of the cholangiocytes expresses ACE2 receptors even though they occupy only 3% to 5% of the liver cell population[27]. Acute liver injury was common in 15.4% of Chinese patients with COVID-19 illness[1]. However, it has been noted that the liver is involved in roughly 60% of cases, and the likelihood of liver malfunction appears to rise with age. A report by Ji *et al*[22] on 202 COVID-19-positives showed that 50% of the patients had some form of liver abnormalities upon admission, and 75% of patients developed liver dysfunction during the course of their stay in the hospital. Most of the liver injury was mild, and only 3% of the patients had ductular or mixed patterns of liver abnormalities. Male gender, older age > 60 years, a high BMI, underlying comorbidities, and MAFLD were all linked to COVID-19 development[28]. MAFLD was identified as having an OR of 6.4 with a 95% confidence interval (CI) of 1.5 to 31.2 in this study by multivariate logistic regression analysis[29]. However, this survey has limitations due to the small number of cases that were available, various severity criteria, underlying comorbidities, and unclear liver disorders[22,30-32]. In contrast, the presence of intermediate or high fibrosis-4 (FIB-4) scores significantly and independently enhanced the probability of severe COVID-19 illness in a sample of 310 individuals with COVID-19 and MAFLD[33]. Due to their increased metabolic risk, patients with MAFLD exhibit a distinct risk[23-26,34].

ACE2 receptors are required for the spike viral proteins to attach to the target cells in order for the COVID-19 disease to progress to its first stage[12]. These receptors are mainly expressed on alveolar epithelial cells (type II) and ciliated cells in the human lung, as well as on the epithelia of the upper respiratory tract (nasopharynx), which is a key site of replication[12]. The vascular endothelium, the brush border of intestinal enterocytes, and cholangiocytes express the ACE2 receptor[14]. Therefore, COVID-19 may cause symptoms to appear in the gastrointestinal tract[15].

According to a recent United States survey, 61% of people who tested positive for COVID-19 had clinically obvious gastrointestinal symptoms[16]. Because ACE2 receptors are found in the glandular cells of the stomach, duodenum, and distal enterocytes, their presence may cause malabsorption, imbalanced intestinal secretion, and enteric nervous system activation, all of which can result in gastrointestinal symptoms[12].

COVID-19 AND LIVER BIOCHEMISTRY PATTERNS AND FREQUENCY

Although the specific impact of COVID-19 on the liver is yet unknown, patients with COVID-19 frequently experience liver biochemistry abnormalities, affecting 15%-65% of SARS-CoV-2-infected people[35-42]. The large variation in these reported frequencies may be due to various interpretations of what constitutes the upper limit of normal, variable lab results regarded as liver enzymes and regional variations in the prevalence and nature of the underlying chronic liver disease (CLD). An estimated 29%-39% and 38%-63% of patients, respectively, have been reported to have mild (1-2 times the upper limit of normal) elevations of their serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, which characterize liver biochemistry abnormalities in COVID-19[36,38,43]. Although severe liver damage, increased blood bilirubin levels, and hepatic synthetic malfunction are all uncommon in SARS-CoV-2 patients, hypoalbuminemia, a non-specific index of illness severity, has been linked to worse COVID-19 outcomes. Non-specific findings from liver biopsies in SARS-CoV-2 patients have included steatosis, moderate lobular and/or portal inflammation, and vascular pathology [10,44,45].

The majority of the time, abnormal biochemistries are likely multifactorial, with direct infection of hepatocytes as well as immune-mediated inflammatory response, drug-induced liver injury, hepatic congestion, and extrahepatic release of transaminases all having a potential role. Elevations in serum AST levels among COVID-19 hospitalized patients positively correlate with levels of ALT but not with

markers of systemic inflammation [such as C-reactive protein (CRP) and ferritin] or muscle breakdown (such as creatinine kinase)[21]. Despite the rarity of reports of rhabdomyolysis (muscle breakdown) related to COVID-19[46], these findings suggest that high liver enzymes in COVID-19 are the result of direct hepatic injury. Finally, during COVID-19, AST is frequently higher than ALT, which is unusual for a classic hepatocellular pattern of liver injury outside of specific situations like alcohol-related liver disease, some drug-induced liver injuries (like those caused by lamotrigine), ischemic hepatitis, and cirrhosis. The causes of an AST-predominant aminotransferase increase are not fully understood, although they may include mitochondrial failure linked to COVID-19, hepatic steatosis brought on by SARS-CoV-2, and altered hepatic perfusion brought on by microthrombotic disease[34,47,48].

EFFECTS OF COVID-19 ON MAFLD DISEASE PROGRESSION

Comorbidities associated with MAFLD

MAFLD is a serious public health issue and a leading cause of CLD globally. MAFLD has been identified as a hepatic manifestation of an insulin resistance-related metabolic syndrome. A growing body of research evidence suggests that systemic disorders like type 2 diabetes, obesity, metabolic syndrome, chronic kidney disease, and cardiovascular disease are all linked to MAFLD. The primary cause of death in MAFLD patients is cardiovascular disease. Rather than just being steatosis, these findings are intricately linked to nonalcoholic steatohepatitis (NASH). MAFLD should be seen as an early mediator of systemic disease in addition to being a liver-specific condition. In relation to other medical illnesses, the pathophysiology, and underlying processes of MAFLD are still poorly understood. Future therapeutic approaches for MAFLD require more research[49]. The various risk factors associated with severe COVID-19 in patients with MAFLD are enumerated in Table 1.

Systemic inflammation and hypoxia

Patients with COVID-19 infection who have chronic liver disorders may express more ACE2 receptors and hypoxia-inducible factors (HIFs), a class of transcription factors triggered by hypoxia[50]. The progression of metabolic illnesses like MAFLD may be accelerated by such changes[31,35-37,39,51-55]. Clinically, biliary ductal abnormalities are uncommon in COVID-19-infected patients; as a result, the ACE2-mediated liver injury may primarily result from the localization of these receptors in endothelial cells. Additionally, the progression of MAFLD involves increased production of reactive oxygen species and nitric oxide derivatives, inflammatory pathways that result in cellular communication with Kupffer cells, and upregulation of HIF through suppression of fatty acid oxidation. This theory is somewhat corroborated by liver histology from patients who died from severe COVID-19, which showed minor lobular and portal activity as well as moderate microvesicular steatosis, presumably as a direct result of SARS-CoV-2 infection or drug-induced liver injury[51,55,56].

Altered liver response

Lipids, which are part of the cell membrane, exosomes, and energy storage components, are strongly associated with the viral life cycle. Infected cells typically have changes in their metabolism of circulating lipids[41,43]. In order to facilitate their replication, viruses alter lipid metabolism, including the expression and activity of crucial enzymes involved in lipid biosynthesis. Changes in lipid metabolism may also be linked to the host's reaction to an infection. SARS-CoV-2 is not an exception and causes significant modifications in lipid metabolism after infection[42,43,57]. SARS-CoV-2 infection specifically causes a general down-regulation of approximately 100 serum lipids, including fatty acids, sphingolipids, and glycerophospholipids. Lipids are not only altered in COVID-19, but they are also linked to pathophysiology and the development of the illness. Changes in bilirubin and bile acids provide evidence that the observed down-regulation of lipids during SARS-CoV-2 infection is related to liver damage. Many of the COVID-19 lipid and lipoprotein changes that have been reported are connected to hepatic activities. The investigation of plasma lipidomic analysis was conducted during COVID-19. Sphingomyelin and monosialodihexosyl ganglioside levels were upregulated, and diacylglycerol levels were downregulated, accounting for the majority of the significantly altered lipids. The severity of the condition was positively linked with higher monosialodihexosyl ganglioside levels. Again, disruption of the normal circulating lipid profiles may be caused by inflammation and infection. Unsaturated fatty acids may be released as a defense mechanism in response to a cytokine storm. When COVID-19 illness is present, proinflammatory lipids and lipid mediators may modify the immunological response[43,57,58]. In addition to the lipid metabolism, liver detoxification and protein synthesis are significantly impaired in the COVID-19 patient. In an autopsy study, looking at the transcriptome of the severe COVID-19 patient with non-covid patient, the cytochrome P450 gene - *ACAD11, CIDEB, GNMT* and *GPAM* were significantly down regulated[59]. This consequently affects the detoxification of drugs and metabolites through the CYP 450 system. The liver is the powerhouse of protein synthesis. It does not only synthesize the anabolic proteins but it also synthesizes proteins involved in both innate and acquired immune responses. This is very much reduced in the MAFLD patient who are in a state of immune dysregulation but the exact role of each component of hepatic immune dysregulation to

Table 1 risk factors associated with severe coronavirus disease 2019 in metabolic-associated fatty liver disease patients

Common risk factors for severe COVID-19 infections	
Obesity	High serum IL-6 at admission
Advanced age > 65 yr	Male gender
Black race	High ferritin level at admission
Liver fibrosis	High EWS at admission
Dyslipidemia	Type 2 diabetes mellitus

EWS: Early warning score; IL-6: Interleukin-6; COVID-19: Coronavirus disease 2019.

COVID-19 severity is difficult to delineate[3].

Liver steatotic state/ lipid derangement

The development of steatosis and liver fibrosis in MAFLD patients is facilitated by active innate immunity in the infectious state, which not only directly causes and intensifies liver inflammation but also interferes with the control of lipid metabolism. In COVID-19 patients, proteomic and metabolomic analysis identified dyslipidemia, including lipid build-up and downregulation of apolipoproteins[57]. In turn, it was discovered that SARS-CoV-2 infection can alter lipid synthesis and absorption pathways, increasing the accumulation of lipid droplets (LD) in human cells[43]. SARS-CoV-2 can also highjack LDs to increase its ability to replicate. Recent research has shown that ACE2 is crucial for maintaining metabolic homeostasis from a mechanistic perspective. A SARS-CoV-2 infection reduces ACE2 expression, which leads to aberrant metabolic processes. Patients with COVID-19 may experience MAFLD development as a result of the metabolic imbalance brought on by ACE2 deficiency[27,42,43,57,58,60].

ELEVATED FIB-4 AND POOR COVID-19 OUTCOMES

A straightforward, thoroughly tested point-of-care measure called the FIB-4 index is used to categorize individuals with suspected MAFLD according to their likelihood of developing liver fibrosis. It uses a combination of patient's age, ALT, AST, and platelet count, all of which may be quickly determined by front-line healthcare professionals[33]. FIB-4 is helpful in identifying liver disease patients who are more likely to experience a negative clinical outcome connected to the liver. FIB-4 has also been demonstrated to predict non-liver clinical outcomes in MAFLD patients, such as cardiovascular mortality or risk of atrial fibrillation. Similarly, FIB-4 has been used to predict mortality in the general population as well as clinical outcomes in clinical situations unrelated to the liver. In the study of Ibáñez-Samaniego *et al*[33], increased FIB-4 Levels were linked to a poor outcome in COVID 19 patients.

The chance of developing an enhanced inflammatory response, a feature of severe COVID-19, may be increased by advanced hepatic fibrosis. Advanced liver disease is actually characterized by a persistent stimulation of immune cells by pathogen-associated molecular patterns and damage-associated molecular patterns[58]. This stimulation causes immune cells to become activated and increases the production of cytokines, chemokines, and growth factors. These growth factors are then released to attract and activate additional inflammatory cells, maintaining a state of chronic low-grade systemic inflammation[33]. Patients with obesity and insulin resistance have been noted to experience a similar level of low-grade inflammation. In fact, the degree of obesity and the likelihood of developing type-2 diabetes mellitus have been linked to increased serum levels of Interleukin-6 (IL-6)[32]. Activated macrophages release IL-6 during an acute infection, which is a significant inducer of the creation of acute phase reactant proteins in hepatocytes (CRP, ferritin, complement, clotting factors). The hepatocytes' acute phase proteins have a direct effector role on innate immunity, facilitating pathogen clearance[21,33,46,61-65].

Increased hepatic decompensation rates in cirrhotic patients

Data on decompensated cirrhosis and COVID-19 is limited. In the first 152 cases of clinically and laboratory-confirmed COVID-19 infections with CLD in two international reporting registries (n = 103 with cirrhosis and n = 40 with chronic liver disease) (COVID-Hep.net and COVIDCirrhosis.org)[66], the probability of death after hepatic decompensation during COVID-19 was significantly higher in those with new decompensation: 63.2% died compared to 26.2% in those without new decompensation. Notably, 24.3% of people with new hepatic decompensation at the time of diagnosis had no pulmonary symptoms of COVID-19. Therefore, decompensated liver disease is a significant risk factor for mortality in COVID-19 patients. As a result, all patients with decompensated liver disease should be hospitalized,

and any recent decompensation in a cirrhotic patient should be tested for COVID-19 at this time[13,66]. In a metanalysis of observational studies of COVID-19 infection with cirrhosis, the patient with cirrhosis not only had higher rate of decompensation but the odds for mortality has been 2.48 (CI 2.2-3.04) when compared to the non-cirrhotic patients[67].

EFFECTS OF PRE-EXISTING MAFLD ON COVID-19 DISEASE SEVERITY

Coagulopathy

Proinflammatory cells may produce cytokines, which can increase the synthesis of procoagulant molecules like tissue factor and von Willebrand factor. This can result in a hypercoagulable condition, which can lead to widespread micro-/macrovascular thrombosis[63]. In addition to elevated levels of tissue factor and von Willebrand factor in the bloodstream, MAFLD patients also have increased levels of platelet activation and plasminogen activator inhibitor type 1 concentration. Patients with COVID-19 who have MAFLD have greater levels of circulating D-dimer than patients without MAFLD, indicating that the pro-coagulant condition associated with MAFLD may be a factor in the severity of COVID-19 [65]. According to findings from a retrospective investigation on a group of COVID-19 patients, people who presented with deep vein thrombosis, confirmed by Doppler ultrasound, had a greater prevalence of MAFLD[46,62]. In addition, COVID-19 patients with MAFLD had a higher mean admission and peak serum D-dimer concentrations than those without MAFLD[46]. In MAFLD individuals, COVID-19 may potentially further boost the production of proinflammatory cytokines, resulting in the activation of the coagulation cascade and thrombosis. In fact, a pathologic analysis of the pulmonary arteries in COVID-19 patients revealed extensive thrombosis with microangiopathy in addition to hepatic steatosis affecting 50%-60% of the liver parenchyma[68]. Hepatic steatosis and pulmonary thrombi were discovered in 55% and 73%, respectively, of COVID-19 patients, according to an Italian post-mortem examination, which corroborated this report[32]. These findings strongly imply a connection between these disorders, with the proinflammatory hypercoagulable state acting as a common pathogenic pathway to severe COVID-19, which promotes thrombosis and the spread of the disease[62-64].

Cytokine production

Prolonged and significant lymphopenia, an abnormal inflammatory response related to aberrant and uncontrolled cytokine activation, and lung mononuclear cell infiltration are all associated with COVID-19[52]. The prognosis of an illness depends on the degree of involvement of additional organs. In fact, observational studies showed that increased levels of inflammatory markers in the blood (CRP, ferritin, and D-dimer), a higher neutrophil-to-lymphocyte ratio, as well as elevated levels of inflammatory cytokines and chemokines were linked to the disease severity and a poor prognosis[21]. One element of liver damage in COVID-19 may be dysregulation of the innate immune response. Inflammatory indicators, such as abnormally high levels of CRP, lymphocytes, neutrophils, and cytokines, are usual in COVID-19 patients[3]. Due to the loss of control over cytokine regulation, pulmonary and extrapulmonary damage occurs. During the early stages, this control could help to slow the evolution of the disease[62]. Hypercytokinemia, that is deadly or fulminant, may set off a series of events that damage or fail many organs, including the liver. Jaundice, hepatic encephalopathy, hepatomegaly, and increased blood transaminase levels could be brought on by the inflammatory response[62]. Since COVID-19 is associated with cytokine storm there is overlap of cytokines involved in both the disorders, however, it will be difficult to point out that these are sole causative agents for hepatic decompensation in MAFLD as there are more factors in play than the cytokines alone. It is also interesting to note that MAFLD patients had a distinct cytokine profile with higher concentrations of IL-6, IL-8, IL-10, and IFN-β when compared to the patients without MAFLD[69]. Higher levels of IL-8 and IL-10 are associated with the worst prognosis and delayed time to recovery[69].

Obesity

Apart from diabetes, the presence of an "overfat" condition (excess body fat that harms health) has become a global pandemic and can occur in obese, overweight, and even normal weight subjects with excess fat involving the liver in the form of steatosis[70]. Several abnormalities can cluster together with overfat, including obesity, overweight, chronic "metabolic" inflammation, and insulin resistance, ultimately configuring the metabolic syndrome[18,20,30,32,44,45]. As seen by the higher prevalence of both autoimmune and immunological illnesses, excess body fat may impede the immunity[41]. Adiposity underlies a compromised immune response (mostly mediated by T cells and macrophages) that increases the risk of infections and chronic respiratory illnesses. Notably, being overweight appears to increase the risk of contracting contagious viral infections[47,48,71,72]. In particular, being overfat may have a negative impact on the immune system performance and host defense mechanism, while being overfat causes hosts to respond improperly to viral and bacterial attacks[48].

The association between obesity and COVID-19 severity persisted after adjusting for age, sex, smoking habits, diabetes, hypertension, and dyslipidemia. In MAFLD patients, obesity was associated with a 6-fold increased risk of severe infection[72]. Patients with MAFLD, especially those who are

obese, have been found to have higher levels of IL-6, which has been linked to an aggravation of the COVID-19 infection[72-74].

Pre-existing CLD

Investigations on COVID-19 rarely include patients with pre-existing liver illness, and these patients' features according to their Child's status or model for end-stage liver disease (MELD) score have not been independently assessed in these studies. The innate immune response against the virus likely caused important changes in the liver enzymes and coagulation profile in 63 patients with severe COVID-19 disease, and CLD was not proven to affect the severity of COVID-19[3]. Cirrhosis patients are now recognized as an independent predictor of COVID-19 severity and a higher hospitalization risk [61]. According to the Child Turcotte Pugh score or MELD score, patients with cirrhosis have a greater mortality rate, and this rate increased as the severity of the liver disease grew[66]. Patients with cirrhosis had a significant 30-d death rate and a 20%-30% chance of decompensation manifesting as acute on chronic liver failure[66]. Data on patients with COVID-19-associated autoimmune hepatitis, chronic viral hepatitis, and alcoholic liver disease are few. Immunosuppressive medications should not be reduced for immunosuppressed patients, whether for autoimmune hepatitis or post-transplantation, out of concern for COVID-19[75]. Data on post-transplant patients are scarce, although there is no evidence of significant COVID-19-related mortality during the peri-transplant period[76]. Routine endoscopy and liver biopsy should be avoided, but urgent procedures for variceal bleed and cholangitis should be carried out according to the correct protocols designed for COVID-19 patients. Various drug combinations are being used with varying degrees of success in treating COVID-19 in patients with cirrhosis. According to the combined findings of the preliminary COVID-19 data, CLD had a negligible impact on patient progression to the severe stage of the disease. However, further research conclusively demonstrated that underlying CLD was associated with worse outcomes and more severe COVID-19 illness[48].

HEPATIC IMMUNE MODULATION

Disconcerting, the inherent chronic activation of inflammatory pathways in MAFLD appears to increase liver damage in patients with COVID-19, perhaps worsening outcomes in those with prior comorbid metabolic disorders. The likelihood of developing more severe types of COVID-19 infection has also been reported to be increased in people with pre-existing chronic liver disorders[23]. Patients with severe COVID-19 infection had high levels of inflammatory markers like CRP, serum ferritin, lactate dehydrogenase, D-dimer, and interleukins (IL-6, IL-2)[36]. Individuals with MAFLD have been found to have elevated IL-6 Levels. When individuals with COVID-19 infection experience a "cytokine storm," IL-6 is a key player. Particularly, IL-6 seems crucial in the beginning and development of the "cytokine storm" seen in COVID-19-infected patients[52]. Elevated IL-6 Levels are linked to MAFLD, which may be a marker or mediator of the comorbidities and related atherosclerosis that are typically observed in COVID-19-infected patients[23]. The cytokine, monocyte chemoattractant protein (MCP-1), is commonly raised in patients with COVID-19 infection, which exacerbates steatohepatitis[23,24,77]. The interplay between COVID-19 and MAFLD in modulating the pathophysiology and outcomes of either disease is shown in Figure 1.

DIAGNOSTIC CHALLENGES

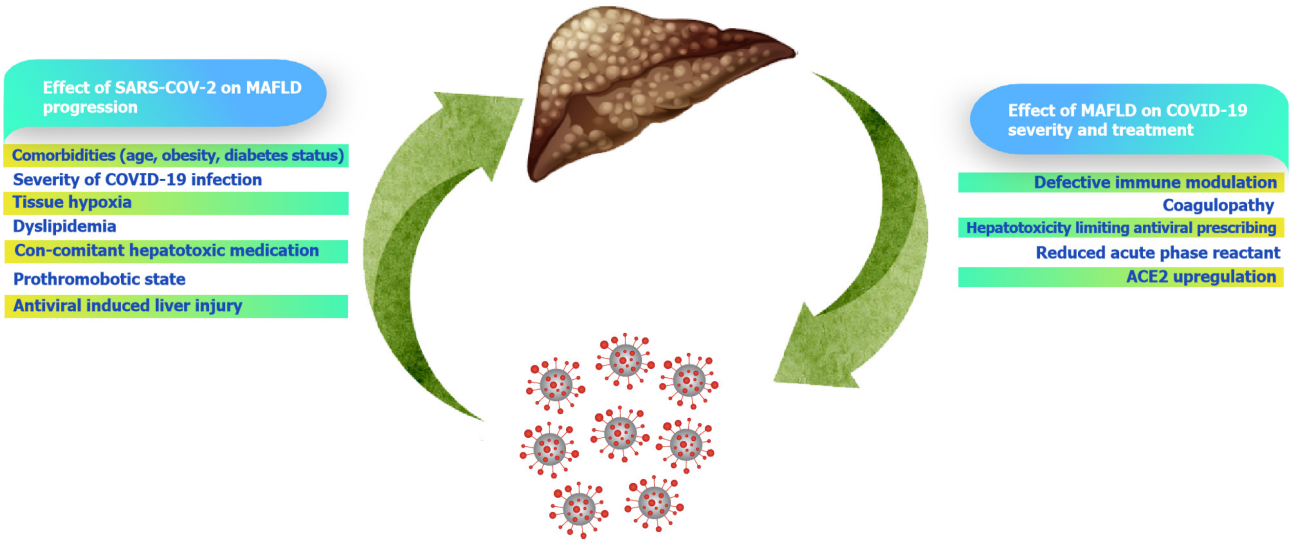
An accurate clinical history, radiographic and laboratory tests, and histologic data are all necessary to diagnose MAFLD. In the absence of significant alcohol intake, other hepatic steatosis-causing factors, and the presence of other liver illnesses, hepatic steatosis must be present in order to diagnose the disease. MAFLD, diagnosed with abdominal imaging, reduces the need for invasive tests like liver biopsies[38]. A liver biopsy may be helpful when deciding between basic steatosis (NAFL) and NASH. Also, a liver biopsy can help in assessing the likelihood of other conditions that will worsen the MAFLD. Patients with COVID-19: Liver biopsy should be postponed in most patients because: (1) Liver biopsy may pose a risk for viral transmission (although the virus has not yet been detected in the liver tissue), the expression of its receptor on cholangiocytes suggests that the virus might be present[49]; (2) COVID-19 treatment/care outweighs diagnosis of concurrent liver disease; and (3) Systemic inflammation associated with COVID-19 will obscure etiology-specific histologic characteristics.

The patient's clinical history must be considered while interpreting test results (Table 2). The "World Gastroenterology Organization" suggests the following guidelines for treating people with liver disease generally in the COVID-19 era[49]: (1) Routine outpatient testing of liver biochemistry is not advised in the COVID-19 era; (2) Discard viral hepatitis in patients with increased ALT or AST levels. Due to the possibility that patients in developing nations have never undergone testing, this may be very crucial; (3) Local context and availability should be considered throughout routine investigation to rule out

Table 2 Analysis of liver test results in coronavirus disease 2019 patients

Test	Comments
Prolonged INR or thrombocytopenia	In one-third of sick patients
	Spontaneous coagulopathy/DIC may be present
	Thromboembolic incidents are probably frequent
	There may be a chance of ACLF
Imaging	Where chest-CT is frequently performed: Assessing liver/biliary tract disease might be helpful
	Do US, if necessary, but refrain from using US for superfluous imaging (not formally investigated)
Hypoalbuminemia	Common in people with systemic inflammatory response
	May also be a sign of acute hepatic decompensation or acute liver failure in people with pre-existing liver cirrhosis
High transaminases or bilirubin (> 3 × ULN)	Although not typical for COVID-19, ACLF may be present in patients with cirrhosis who already have liver disease
Dyselectrolytemia	Diarrhea and other GI problems might result in numerous electrolyte abnormalities
Anemia	Consider bleeding due to variceal hemorrhage in the context of MAFLD cirrhosis, portal hypertensive gastropathy or stress mucosal GI ulcer

ACLF: Acute on chronic liver failure; CT: Computed tomography; DIC: Disseminated intravascular coagulation; GI: Gastrointestinal; ULN: Upper limit of normal; US: Ultrasonography; COVID-19: Coronavirus disease 2019; MAFLD: Metabolic-associated fatty liver disease.



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Figure 1 Interplay between coronavirus disease 2019 and metabolic-associated fatty liver disease. COVID-19: Coronavirus disease 2019; MAFLD: Metabolic-associated fatty liver disease; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

other etiologies; and (4) Regular imaging should only be done if it will change management.

NONINVASIVE MARKERS FOR FIBROSIS DETECTION

The increasing prevalence of MAFLD, the limits of liver biopsy, and the lack of consensus regarding clinical predictors of NASH have generated a market for next-generation noninvasive biomarkers and imaging modalities to aid in the distinction between MAFLD and NASH. Aminotransferases, cytokeratin-18, and numerous scoring systems that incorporate laboratory indicators such as the AST/platelet ratio index, NAFLD fibrosis score, FIB-4 index, and Fibrotest are some examples of indirect markers. The extracellular matrix contains direct fibrosis indicators such as fibronectin, elastin, laminin, and hyaluronic acid which develop in the presence of prolonged hepatocyte damage and have also been included in certain ratings[33,78].

ENDOSCOPY FOR PATIENTS WITH COVID-19

Patients who are at risk of variceal bleeding, such as those with a history of variceal bleeding or symptoms of significant portal hypertension (ascites, low thrombocyte count, ALT > 5 × ULN) of unknown etiology, should be considered for esophago-gastro-duodenoscopic variceal screening (in case of suspected autoimmune liver disease, treatment without a histological diagnosis can be considered based on individual risk-benefit considerations)[49,75].

DETECTION OF ACE2 POLYMORPHISM

Studies on whether *ACE1/ACE2* genetic variability influences the clinical course of COVID-19 in diverse ethnic communities remain elusive[14]. Between Asians and Caucasians, *ACE2* demonstrated significant minor allele frequency differences due to four missense mutations[71]. 64 K26R and I468W, two of these variations, may influence how the S protein of SARS-CoV-2 binds to the hACE2 receptor[55]. A difference in male and female individuals was found in *ACE2* expression between Asians and others [79]. The *ACE2* variant rs2285666 was not connected to the course of the disease when *ACE2* genetic variation was examined in the COVID-19 progression[71]. Nevertheless, numerous studies have shown a substantial correlation between COVID-19 and *ACE1*-insertion/deletion (I/D)[71,77,80]. When compared to *ACE1-II* people, *ACE1-DD* carriers had higher blood levels of ACE-I that are roughly twice as high and have been linked to hypertension, ARDS, and in-hospital mortality[81]. As a result, although the *ACE1-II* genotype negatively correlates with infection rate and mortality, the deletion allele positively corresponds with COVID-19 progression and SARS-CoV-2 infection rate and mortality. However, *ACE1-I/D* allele frequency ratio was substantially linked to the rise in recovery rate but not to mortality in a meta-analysis of 48758 healthy adults from 30 different nations[82]. Additionally, *ACE1-I/D* polymorphisms may help to explain how COVID-19 manifests in different ethnic populations. African Americans (29%, 60%, and 11%, respectively), Indians (19%, 50%, and 31%, respectively), and Whites (29%, 40%, and 31%, respectively) all had statistically different distributions of the *D/D*, *I/D*, and *I/I* genotype frequency ratios[82]. Additionally, there was a statistically significant difference in the frequency of the deletion allele among African Americans, Indians, and whites (0.59, 0.49, and 0.44, respectively)[79,81]. More research is necessary to determine whether these indications could explain COVID-19 progression in various populations. Overall, more *in vitro* and functional research are needed to fully understand the importance of the *ACE* and *ACE2* allele frequency ratio findings and how they relate to the COVID-19 studies[71]. These investigations ought to look into the morbidity and mortality hazards linked to COVID-19 and MAFLD in these racially varied genetic variants.

CLINICAL INTERVENTIONS AND MANAGEMENT

Antivirals and monoclonal antibodies

There is a paucity of information on the safety and effectiveness of new and existing COVID-19 treatments in patients with MAFLD, CLD, and cirrhosis. Clinical professionals' key worries are around adverse immune-related events, long-term effects, and drug interactions. On the basis of the presumption that dysregulated immune responses need to be suppressed; a number of medicines have been evaluated in COVID-19. Steroids, such as dexamethasone, prednisolone, methylprednisolone, or intravenous hydrocortisone, which act through the glucocorticoid receptor and effector genes, are one of the principal treatments. According to the World Health Organization recommendations; systemic corticosteroid medication is not recommended for everyday usage[5,83]. Only patients who have cytokine storm, ARDS, severe cardiac failure, acute kidney injury, and high serum D-dimer levels should receive it. Janus Kinase JAK inhibitors, IL-1 and IL-6 inhibitors, anti-tumor necrosis factor-alpha (often referred to as anti-TNF-alpha) medications, corticosteroids, colchicine, and intravenous immunoglobulin are other immunomodulators studied in COVID-19 infection[83]. Chloroquine and hydroxychloroquine have been shown to lessen COVID-19-mediated damage by stopping the cytokine storm, activating CD8+ cells, or blocking the virus from being taken up by endocytosis[84]. By building up in lysosomes and raising the pH of the endosome, chloroquine and hydroxychloroquine block the entry and departure of viruses from cells. Additionally, these medications block the ACE2 receptor, inhibiting SARS-CoV-2 entrance. Chloroquine and hydroxychloroquine may lessen the ACE2 receptor's glycosylation, preventing the virus from attaching to and infecting new cells. Chloroquine and hydroxychloroquine have been known to cause QT prolongation due to a delay in the cardiomyocyte depolarization rate[85]. There have been reports of patients developing torsades de pointes with the use of chloroquine[86]. Major studies, however, failed to demonstrate any alleged COVID-19 prophylactic and therapeutic benefit, and these medications have subsequently fallen out of favor due to their serious cardiovascular complication risk[87]. Other direct antivirals, like remdesivir and favipiravir, similarly did not demonstrate any appreciable efficacy or survival advantage[83]. In COVID-19, tocilizumab, a

Table 3 Drugs and vaccines used in the management and prevention of coronavirus disease 19

Classification	Drugs
Antiviral agents	Favipiravir, molnupiravir, paxlovid, remdesivir
Immunomodulatory agents	
JAK inhibitors	Baricitinib, ruxolitinib, tofacitinib
Monoclonal antibodies to IL-6	Sarilumab, tocilizumab
Corticosteroids	Cortisol, dexamethasone, methylprednisolone
Monoclonal antibodies to SARS-CoV-2	Bamlanivimab, casirivimab, etesevimab, imdevimab, sotrovimab
COVID-19 vaccines	
mRNA	BNT162b2 [Pfizer-BioNTech], mRNA-1273 [Moderna]
Adenovirus vector	ChAdOx1-S [AstraZeneca, Oxford]; Ad26.COV2.S [Johnson and Johnson, Janssen], Sputnik-V-Gam - COVID Vac Ad5+Ad26 [Gamleaya]
Recombinant nanoparticles	NVX-CoV2373 [Novavax]
Miscellaneous agents	Azithromycin, chloroquine, dexamethasone, fluvoxamine, hydroxychloroquine, ivermectin

COVID-19: Coronavirus disease 19; IL-6: Interleukin-6; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

humanized IgG1 monoclonal antibody to the IL-6 receptor, has shown only patchy success. However, the adverse effects can include hepatotoxicity, diverticulitis, hypertriglyceridemia, and increased susceptibility to infection[88]. Cytokine dialysis, utilizing blood ultrafiltration, diffusion, and adsorption circuits in dialysis machines, has also been tested as an alternative to medications that directly decrease the immune response[44]. Theoretically, restoring immunological IL-6/IL-1 levels and other proinflammatory molecules protect against organ failures, but the clinical effectiveness of this protection is still unknown, and immune dysregulation is just one issue among many[89]. It has been suggested to use immunomodulators based on mesenchymal stem cells to prevent and control the cytokine storm. Mesenchymal stem cell transplantation intravenously was proven successful in COVID-19 patients in a study[90].

Of the drugs listed in Table 3, the commonly used agents and their hepatoxicity profile are shown in Table 4. The appropriate selection of drugs in MAFLD patients depends on the severity of COVID-19 infection, duration of the disease, ALT level, and potential drug interactions with other medications. Our approach is in line with the Infectious Disease Society of America 2022 guidelines and is shown in Figure 2[91]. Hence when these drugs are used, liver function tests should be routinely monitored, and manufacturers' advice regarding dose adjustment should be followed until more studies are available in MAFLD patients. A very rare case of acute severe hepatitis with the use of Tocilizumab was noted in a patient who had previous lopinavir and ritonavir exposures[88]. In general, the management of these drug induced liver injury is usually symptomatic and in severe cases, when the ALT is more than six times the upper limit of normal, the medication may need to be temporarily stopped[44].

Vaccines

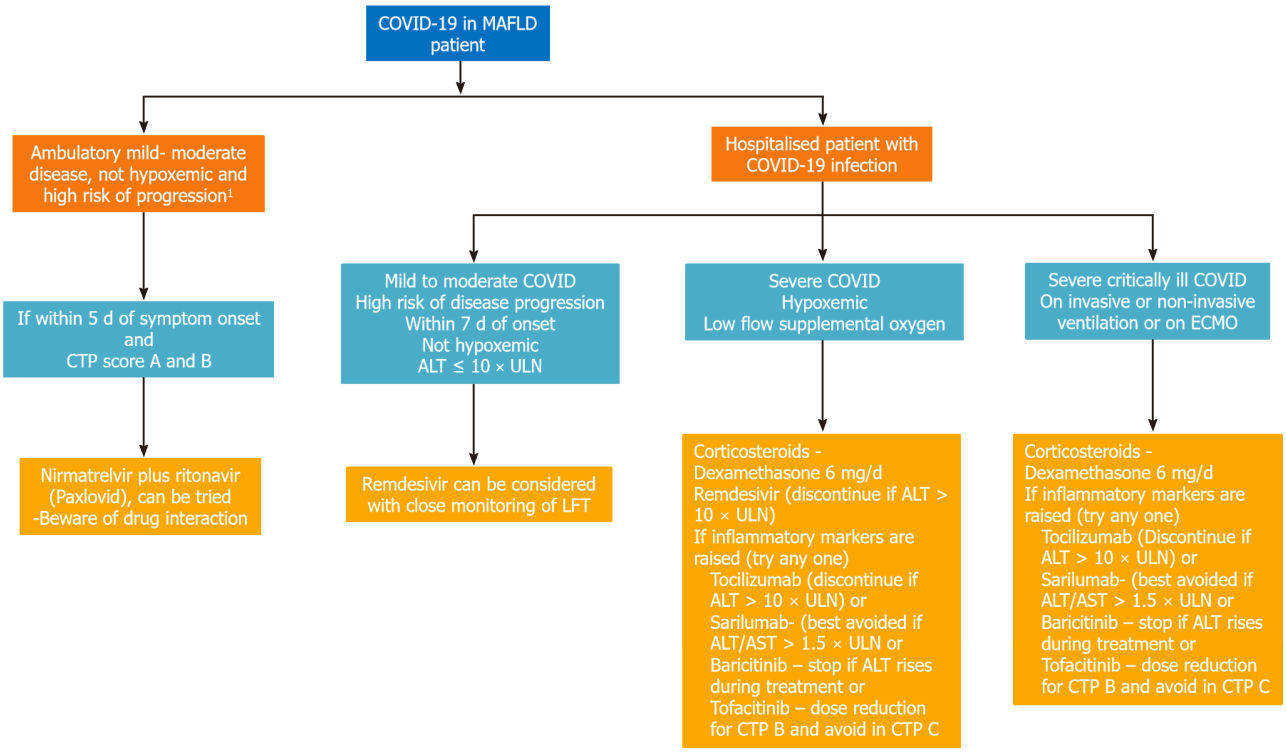
At least 85 vaccine proposals were being researched in clinical trial phases, and 184 vaccines were being evaluated in pre-clinical stages, according to the most recent version of the WHO report from April 2, 2021[28]. Other vaccines, including the plant-derived vaccine and the *Bacillus Calmette-Guérin* vaccine, have also been proven in tests to potentially aid in the management of the COVID-19 pandemic[92].

The currently commercially available vaccines include Oxford-AstraZeneca, Pfizer-BioNTech, Moderna, Sinopharm-Beijing, Gamaleya (Sputnik V), Sinovac, Sinopharm-Wuhan, Johnson & Johnson, Bharat Biotech (Covaxin), CanSino and Vector Institute (EpiVacCorona). In the multicenter study conducted by Wang *et al*[93], in patients with MAFLD who had two doses of inactivated vaccine against SARS-CoV2 without a history of SARS-CoV-2 infection, these vaccines were found to be safe with good immunogenicity. In a multicentric study from China, the inactivated vaccine induced adequate antibody titer against SARS-CoV-2 in 95% of the patients with MAFLD. The adverse event profile was similar to the individuals without MAFLD and hence the vaccine is safe and equally immunogenic as in the normal population[93].

Table 4 Hepatoxicity profile of the commonly used drugs to treat coronavirus disease 2019 infection

Medication	Hepatotoxicity pattern
Dexamethasone	None reported at the dose given for COVID
Protease inhibitors (*e.g.*, lopinavir, ritonavir)	Mostly hepatitis pattern with ALT raise up to 6 times the normal, but rarely cholestatic pattern reported[43]
Nucleoside analogue: Remdesivir	Hepatitis pattern with mild to moderate ALT raise (up to 6 times the normal)[77]
Monoclonal antibodies to IL-6: Tocilizumab	Rarely can cause acute severe hepatitis in patients on concomitant or previous hepatotoxic drug usage[83]

ALT: Alanine transaminase; COVID: Coronavirus disease; IL-6: Interleukin-6.



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Figure 2 Selection of coronavirus disease 2019 therapy in metabolic-associated fatty liver disease patient. [1]High risk of progression - advanced age ≥ 65-yr-old, immunocompromised state or multiple medical co-morbidities. Hypoxemia: SpO_2 ≤ 94% on room air; ECMO: Extracorporeal membrane oxygenation; Mild disease: Cough, upper respiratory tract symptom and absence of dyspnea; Severe: Hypoxemia or need for supplemental oxygen; Moderate: Dyspneic patient and absence of severe disease features. ALT: Alanine transaminase; AST: Aspartate transaminase; COVID: Coronavirus disease; CTP: Child Pugh score; LFT: Liver function test; ULN: Upper limit of normal.

CONCLUSION

SARS-CoV-2 infection's pandemic traits and high mortality rate have sparked worries about the processes causing harm to vulnerable patients. The people most susceptible to COVID-19 had pre-existing illnesses. As a result of metabolic irregularities, the accumulation of metabolically active fat (also known as the "overfat state") coexists with chronic inflammatory alterations, the emergence of insulin resistance, the buildup of fat in the liver, and perhaps even hepatic fibrosis in the long run. This interplay between the numerous inflammatory pathways constantly present in MAFLD can dramatically increase the risk for COVID-19 infection and intensify liver damage. MAFLD should therefore be considered as a prognostic indication during COVID-19, while on the other hand, close long-term monitoring of individuals with MAFLD who experienced COVID-19 may be required. Finally, reducing the vulnerability to non-communicable diseases and boosting personal resistance to future epidemics are additional challenges in diagnosing and treating individuals with MAFLD.

FOOTNOTES

Author contributions: Jeeyavudeen MS substantially contributed to the conception and design of the article, interpretation of relevant literature, article drafting, revision and figure preparation; Chaudhari R contributed to the interpretation of relevant literature, and article drafting; Pappachan JM and Fouda S contributed to the literature search and revision of the article critically for important intellectual content; All authors have read and approved the final version of the manuscript.

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Country/Territory of origin: United Kingdom

ORCID number: Mohammad Sadiq Jeeyavudeen 0000-0002-1461-0534; Rahul Chaudhari 0000-0002-0265-8100; Joseph M Pappachan 0000-0003-0886-5255; Sherouk Fouda 0000-0001-8227-4571.

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MINIREVIEWS

Potential role of the microbiome in liver injury during COVID-19: Further research is needed

Marcos Roberto Tovani-Palone, Paolo Pedersini

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Marcos Roberto Tovani-Palone, Department of Research Analytics, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences (SIMATS), Chennai 600077, India

Paolo Pedersini, IRCCS Fondazione Don Carlo Gnocchi, Milan 20148, Italy

Corresponding author: Marcos Roberto Tovani-Palone, PhD, Academic Research, Department of Research Analytics, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences (SIMATS), Poonamallee High Road, Velappanchavadi, Chennai 600077, India. marcos_palone@hotmail.com

Abstract

Although different studies have associated coronavirus disease 2019 (COVID-19) with the occurrence of liver injury, the hepatic injury route during the COVID-19 course is not yet fully understood. In order to better understand the mechanisms of the disease, the human gut microbiota has been the subject of extensive discussion in the context of COVID-19 pathophysiology. However, many questions remain, including the risks of liver injury due to COVID-19 specific populations. Further research in this field could allow the discovery of new personalized treatment strategies aimed at improving the microbiota composition, thereby reducing COVID-19 severity and its complications in different populations. In this article, we discussed basic mechanisms of severe acute respiratory syndrome coronavirus 2 infection and recent evidence on the relationship between COVID-19, the gut microbiome and liver injury as well as proposed recommendations for further research.

Key Words: COVID-19; Gut microbiota; Coronavirus; Gut microbial-host-immune axis; Gut-lung axis; Liver injury

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Core Tip: Although different studies have associated coronavirus disease 2019 (COVID-19) with the occurrence of liver injury, the hepatic injury route during the COVID-19 course is not yet fully understood. Further research is needed to better understand the impacts of changes of the gut microbiota and immunology of COVID-19.

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INTRODUCTION

The gut-liver axis is a well-described bidirectional relationship where a mutual interaction between gut and liver microbiota occurs. It has attracted significant attention in the context of coronavirus disease 2019 (COVID-19). This close anatomical and functional relationship between the gut and its microbiota and liver function results from an interaction between genetic and environmental factors, including diet, medicine use and diseases[1]. Although the human gut microbiota is recognized to have an important role for immunity and protection against pathogens, its diversity decreases in old age, which is the age group with the highest mortality from COVID-19[2]. This suggests a potential protection of balanced gut-liver axis against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, which should be of interest to prevent and reduce the number of fatal cases of COVID-19. On the other hand, any imbalance of this microbiome should affect immunity as well as viral activity against SARS-CoV-2 [3]. Moreover, different studies have also reported the occurrence of liver injury to varying degrees in COVID-19 patients, which could be associated with important changes in both the gut-liver axis microbiota and responses at the cellular and molecular level[4,5]. However, research on the risks of liver injury due to COVID-19 in many specific populations is still scarce. Here we discussed basic mechanisms of SARS-CoV-2 infection and recent evidence on the relationship between COVID-19, the gut microbiome and liver injury as well as proposed recommendations for further research.

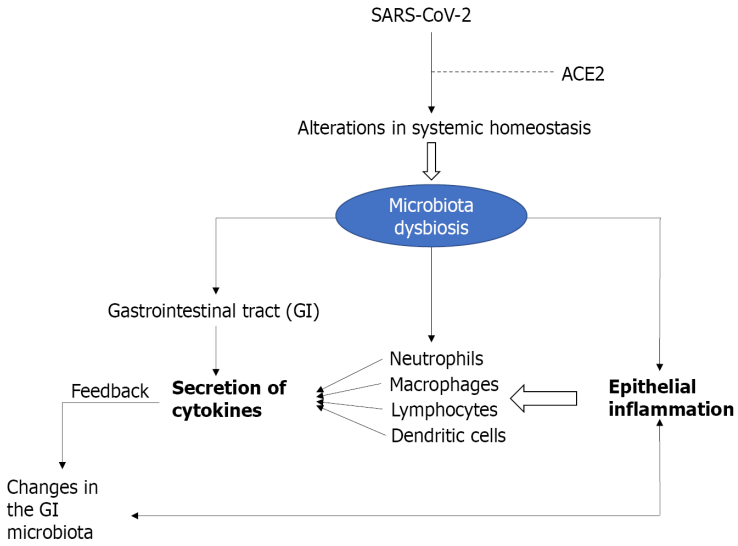
CELLULAR ENTRY OF SARS-COV-2 AND GENERAL IMPLICATIONS

There is a consensus among most scientists that the cellular entry of SARS-CoV-2 primarily occurs *via* high-affinity interactions between the receptor-binding domain of the SARS-CoV-2 spike protein and the angiotensin converting enzyme 2 (ACE2) receptor, in addition to other molecules[4-7]. This receptor has been identified in different and important organs, including the surface of respiratory tract epithelium, epithelial cells of the upper esophagus, enterocytes of the ileum and colon, in the heart, testicles, cells of smooth muscles, the endothelium of pancreatic, brain and kidney blood vessels[4], and in bile duct epithelial cell and liver[5]. The resulting downregulation of ACE2 activity may lead to an increase in angiotensin 2 through ACE. This is due to the fact that the decrease in ACE2 is associated with a lower conversion of angiotensin to angiotensin 1-7 vasodilator. Thus, there is a gradual tendency towards an increase in plasma concentrations of angiotensin I and angiotensin II, causing an imbalance in the renin-angiotensin system as well as a consequent deregulation of systemic homeostasis[6,8].

COVID-19 AND GUT

According to general statistics, about half of COVID-19 patients are expected to have at least one of these gastrointestinal symptoms: diarrhea, nausea, vomiting, and abdominal pain[4,5]. Research has shown that the ACE2 receptor is the main gateway for SARS-CoV-2 into epithelial cells of the gastrointestinal tract. This receptor is in turn highly expressed on epithelial cells in the small intestine. In addition to the decrease in ACE2 receptor expression due to the invasion of SARS-CoV-2, important changes in the gut microbiota involving different microorganisms (dysbiosis) may also occur (Figure 1), affecting the function of the intestinal barrier and the permeability and homeostatic balance of metabolites in the gut lumen[8,9].

It is also hypothesized that SARS-CoV-2 infection of epithelial cells in the gut, especially in the small intestine, could result in malnutrition as well as potentiate the associated dysbiosis, leading to impaired gut barrier function and systemic inflammation. This in turn may create a positive feedback loop for increased translocation of gut microbes into the systemic circulation and potentiation of inflammation, culminating in systemic inflammation and cytokine storm that may contribute to both worsening gut and systemic damage as well as increasing the severity of COVID-19[8,9]. Therefore, in addition to the classic gastrointestinal disorders and symptoms of COVID-19, accessory digestive organs such as the liver can be affected, as a result of the worsening infection[4].



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Figure 1 Pathway of severe acute respiratory syndrome coronavirus 2 to gastrointestinal microbiota imbalance. ACE2: Angiotensin converting enzyme 2; GI: Gastrointestinal tract; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

COVID-19 AND LIVER INJURY

Although COVID-19 has been associated with liver injury, the hepatic injury route during the COVID-19 course is not yet fully understood. It is believed that such injury is due to specific pathogenic mechanisms of the virus or even the use of hepatotoxic drugs[3,4]. Among the different etiological hypotheses described in the literature in order to advance knowledge about this topic the following stand out: (1) Liver injury resulting from a direct virus cytopathic effect by lysis or by inducing apoptosis; (2) Immune-mediated liver injury from proinflammatory cytokines (interleukin-1, interleukin-6, tumor necrosis factor, chemokines, and inflammatory cells produced against SARS-CoV-2); (3) Liver injury resulting from viral-induced cytotoxic T cells (CD8); (4) Liver injury due to the use of drugs including antivirals, anti-inflammatory drugs, anticoagulants, antibiotics, and drugs used for chronic diseases during SARS-CoV-2 infection; (5) Liver injury caused by hypoxia resulting from pneumonia[4,5]; and (6) Liver injury resulting from the gut vascular barrier and dysbiosis due to the indirect effect of toxic compounds from opportunistic microorganisms[5].

COVID-19, THE GUT MICROBIOME AND LIVER INJURY

More specifically, researchers in this field believe that the occurrence of prolonged gut microbiome dysbiosis in COVID-19 patients may be associated with two important phenomena: fecal shedding of the virus into the environment and disease severity. Evidence for this pathophysiological mechanism is based on the hypothesis that dysbiosis may lead to epithelial inflammation and an increase in ACE2 expression. Given that ACE2 plays a key role in dietary amino acid homeostasis, patients can be severely affected. In this connection, SARS-CoV-2 binds to ACE2, leading to microflora imbalance. This is because the possible downregulation of ACE2 may reduce the secretion of antimicrobial peptides and in turn lead to increased pathogen survival and gut dysbiosis[5]. It is also worth noting that some drugs used to treat COVID-19, such as corticosteroids, have been shown to interact with the gut microbiome. This is also true for chloroquine, which has been equivocally administered to many patients[3,5] as well as different medicinal herbs[10].

Despite this, in the current context of the ongoing pandemic, although a large amount of research has been published on liver injury due to COVID-19[4,5], there remain many questions to be answered (Table 1), including the risk of this type of injury in specific populations. Important research has demonstrated a greater vulnerability to alterations in the composition of the gut microbiota in different populations. This is true for example for the population of individuals with cleft lip and palate[11] and Hashimoto's thyroiditis[12]. Therefore, knowing more about interactions between the human microbiota and the host cytokine pathway should be of great relevance. One of the justifications for carrying out further research in this field includes the need to discover new personalized treatment strategies to improve the composition of the gut microbiota in order to more effectively reduce the severity of COVID-19 and its complications[3,5]. This in conjunction with a healthy lifestyle could have positive impacts on both COVID-19 prevention and treatment[13,14].

Table 1 Other important questions to be answered by new research

No.	Major questions
1	Mean duration of dysbiosis associated with liver injury due to COVID-19
2	Differences in the magnitude of liver injury and changes in the microbiota associated with COVID-19 in patients with varying degrees of disease severity
3	The impact of different drugs metabolized in the liver on the worsening of liver injury associated with COVID-19 and changes in the gastrointestinal microbiota
4	Whether changes in the gastrointestinal microbiota and liver injury associated with COVID-19 are also related to long-COVID-19 symptoms
5	Effective medical protocols and/or treatments to prevent changes in the gastrointestinal microbiota and prevent or treat this type of liver injury
6	The impact of healthy habits on the prevention of changes in the gastrointestinal microbiota and recovery of liver injury in COVID-19 patients

COVID-19: Coronavirus disease 2019.

CONCLUSION

In view of the development of new COVID-19 vaccines, another important point to consider is that the microbiome may affect the immune response to vaccines. This is due to the fact that the immunogenicity can be impaired with dysbiosis[5]. Moreover, due to the likely global endemic situation of COVID-19, further microbiological and immunological research may be critical to determine the impact of changes to the balance of the human microbiota and immunology related to COVID-19 in order to achieve better predictions in the fight against possible new SARS-CoV-2 variants.

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FOOTNOTES

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Country/Territory of origin: India

ORCID number: Marcos Roberto Tovani-Palone 0000-0003-1149-2437; Paolo Pedersini 0000-0003-0224-1783.

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MINIREVIEWS

Artificial intelligence and inflammatory bowel disease: Where are we going?

Leonardo Da Rio, Marco Spadaccini, Tommaso Lorenzo Parigi, Roberto Gabbiadini, Arianna Dal Buono, Anita Busacca, Roberta Maselli, Alessandro Fugazza, Matteo Colombo, Silvia Carrara, Gianluca Franchellucci, Ludovico Alfarone, Antonio Facciorusso, Cesare Hassan, Alessandro Repici, Alessandro Armuzzi

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Leonardo Da Rio, Marco Spadaccini, Roberta Maselli, Alessandro Fugazza, Matteo Colombo, Silvia Carrara, Gianluca Franchellucci, Ludovico Alfarone, Cesare Hassan, Alessandro Repici, Department of Endoscopy, Humanitas Research Hospital, IRCCS, Rozzano 20089, Milano, Italy

Leonardo Da Rio, Marco Spadaccini, Tommaso Lorenzo Parigi, Roberta Maselli, Gianluca Franchellucci, Ludovico Alfarone, Cesare Hassan, Alessandro Repici, Department of Biomedical Sciences, Humanitas University, Rozzano 20089, Milano, Italy

Tommaso Lorenzo Parigi, Roberto Gabbiadini, Arianna Dal Buono, Anita Busacca, Alessandro Armuzzi, IBD Center, Humanitas Research Hospital, IRCCS, Rozzano 20089, Milano, Italy

Antonio Facciorusso, Gastroenterology Unit, Department of Medical Sciences, University of Foggia, Foggia 71122, Foggia, Italy

Corresponding author: Marco Spadaccini, MD, Doctor, Department of Endoscopy, Humanitas Research Hospital, IRCCS, 56 Via Manzoni, Rozzano 20089, Milano, Italy.
marco.spadaccini@humanitas.it

Abstract

Inflammatory bowel diseases, namely ulcerative colitis and Crohn's disease, are chronic and relapsing conditions that pose a growing burden on healthcare systems worldwide. Because of their complex and partly unknown etiology and pathogenesis, the management of ulcerative colitis and Crohn's disease can prove challenging not only from a clinical point of view but also for resource optimization. Artificial intelligence, an umbrella term that encompasses any cognitive function developed by machines for learning or problem solving, and its subsets machine learning and deep learning are becoming ever more essential tools with a plethora of applications in most medical specialties. In this regard gastroenterology is no exception, and due to the importance of endoscopy and imaging numerous clinical studies have been gradually highlighting the relevant role that artificial intelligence has in inflammatory bowel diseases as well. The aim of this review was to summarize the most recent evidence on the use of artificial intelligence in inflammatory bowel diseases in various contexts such as diagnosis, follow-up, treatment, prognosis, cancer surveillance, data collection, and analysis. Moreover, insights into the potential further developments in this field and their

effects on future clinical practice were discussed.

Key Words: Inflammatory bowel disease; Artificial intelligence; Machine learning; Crohn's disease; Ulcerative colitis; Computer-aided diagnosis

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Core Tip: Management of patients with inflammatory bowel disease is complex and costly. Therefore, in this field being able to improve clinical efficiency and optimize healthcare resources is of paramount importance. In this regard, artificial intelligence appears to be an extremely promising tool with a significantly wide range of potential applications that encompass diagnosis, treatment, and follow-up. This review summarized the most recent significant scientific findings regarding the application of artificial intelligence in inflammatory bowel diseases, providing a picture of the current state of the field and future perspectives.

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INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic and relapsing conditions that affect the gastrointestinal tract and are labelled as inflammatory bowel diseases (IBD)[1,2]. In the last few decades, IBD has emerged as a global disease with a conspicuous burden on public health and healthcare costs [3].

The diagnosis and management of IBD is complex and implies the interplay and synergy of various specialists, including clinical gastroenterologists, gastrointestinal endoscopists, radiologists, pathologists, surgeons, and clinical nutritionists[4].

Along with histological assessment, endoscopy has an important role in the diagnosis and the follow-up of IBD while also being the mainstay for colorectal cancer surveillance[5]. Due to the essential role of endoscopy and imaging in gastroenterology, and particularly in IBD, artificial intelligence (AI)-based image analysis can be utilized in numerous applications such as evaluation of endoscopic lesions, cancer detection, and assessment of disease activity (*e.g.*, prognosis and response to treatment)[6].

ARTIFICIAL INTELLIGENCE

AI is an umbrella term that encompasses any cognitive function developed by machines for learning or problem solving. A particular subset of AI is represented by machine learning (ML), a discipline that uses large datasets as an input in order to identify patterns of interaction among variables, allowing the possibility to apply these findings to new data[7].

A further subset and evolution of ML is deep learning (DL), which mimics the neuronal interaction in the human brain to develop artificial neural networks and subsequently convolution neural networks (CNN) that are then able to use the input data in an autonomous fashion with the aim of assessing predictive factors of a specific outcome through the development of multiple levels of abstractions[8].

Among the numerous implementations of AI in medicine, a promising field is that of automatic collection of complex and nuanced clinical data from electronic medical records through natural language processing, the subset of AI that studies the interpretation of the human language made by the computer[9,10].

Another fertile ground of application of AI is the interpretation of radiologic imaging. A computer model based on computed tomography enterography allows for accurate characterization of intestinal fibrosis in CD, in some instances outperforming human radiologists[11].

In the context of gastrointestinal endoscopy, AI has found application in two main fields, namely in the detection of mucosal lesions, with computer-aided detection (CADe) and in the characterization of mucosal lesions, with computer-aided diagnosis, along with the evaluation of the quality of the endoscopic procedure itself, with computer aided monitoring[12-20].

Therefore, implementation of AI in IBD is a promising tool for improving the assessment of disease activity and reducing the interobserver variability in grading such activity[21]. In addition, similar to what is already happening in the general population, CADe systems could eventually improve detection of IBD-associated dysplasia[22]. Finally, AI may also allow for application of precision medicine through the analysis of large databases, correlating differences in the biology of the patient with differences in the susceptibility to develop IBD, the activity of the disease, and the response to specific therapies[23] (Figure 1). Such considerations are further supported by the growing number of clinical studies on the application of AI for IBD in recent years (Figure 2).

Table 1 summarized the most impactful studies on AI in several fields of IBD that were identified after a literature review using PubMed (MEDLINE) from inception to November 6, 2022. The impact measure of the articles cited was assessed *via* the *Reference Citation Analysis* (RCA; Baishideng Publishing Group Inc.) tool.

AI IN ETIOLOGY, PATHOGENESIS, AND DIAGNOSIS OF IBD

Although it is not yet thoroughly understood, the etiology of IBD is known to depend upon the complex interplay of genetic, microbial, and environmental factors and the immune system[24]. In this context, AI allows for more effective data analysis to evaluate the role of specific genes in the predisposition and development of IBD.

Genome-wide association studies have been employed in order to identify sequence variations related with specific conditions, with over 200 genes found to be potentially implicated in IBD etiology [25]. Such complex and large genomic data, however, are difficult to assess with standard analytical tools. In this context, studies have shown that ML and DL can effectively analyze genome-wide association study data and overcome part of the inherent limitation of such methodology through algorithms of minimum Redundancy-Maximum Relevance and incremental feature selection[26-28].

Gut microbiota is thought to play a relevant role in the complex etiology and pathogenesis of IBD, partly concurring in providing a substrate that may range from protective to proinflammatory[29]. Moreover, in the context of dysbiosis, the taxonomic composition appears to vary between patients affected by IBD and unaffected subjects and between IBD subtypes, UC, and CD. Promising studies in this field show that the application of ML algorithms on the analysis of gut microbiome data may assist the clinician in diagnosing IBD[30,31].

AI has also been employed in the attempt of supporting the conventional diagnosis of IBD. In this regard, isolated and combined endoscopic and histological parameters were used to develop ML models, which was accurate in the classification of pediatric patients affected by IBD[32].

Additional models based on confocal laser endomicroscopy were developed and by quantitative analysis through cryptometry were able to provide a diagnosis of IBD with high sensitivity and specificity. Moreover, these models managed to successfully differentiate between UC and CD[33].

AI APPLICATIONS FOR ENDOSCOPIC ASSESSMENT IN IBD

In order to rigorously define endoscopic disease activity through specific parameters and limit interobserver variability, a plethora of endoscopic scores have been developed in the field of IBD. The most relevant scores are the CD endoscopic index of severity and the simple endoscopic score for CD, the Mayo endoscopic score, the UC endoscopic index of severity, and the UC colonoscopic index[34-38].

Implementation of AI in this field might be a further step towards reproducibility and homogeneity of endoscopic findings. The first successful attempts in identifying the presence of mucosal remission or activity *via* AI were made using a dedicated CADe system based on CNN trained on large datasets of endoscopic still images in patients affected by UC[39,40].

A subsequent development was represented by the implementation of neural networks in order to assess disease activity not only on still images but on the entirety of colonoscopy videos in real time. Also in this field, AI was efficient in recognizing active disease, calculating scores, predicting the risk of clinical relapse, and supporting clinicians in real time decision making for treatment[41-44].

Moreover, deep neural networks trained on endoscopic images and histological reports of UC managed to identify patients in endoscopic remission and histological remission with such an accuracy that it may potentially obviate the need for biopsy collection and analysis to identify patients in remission[45].

With regards to prediction of *in vivo* histological activity, the first dedicated CADe system was recently developed for application in endocytoscopy, yielding a high accuracy when compared with the gold standard of the pathologist's assessment[46].

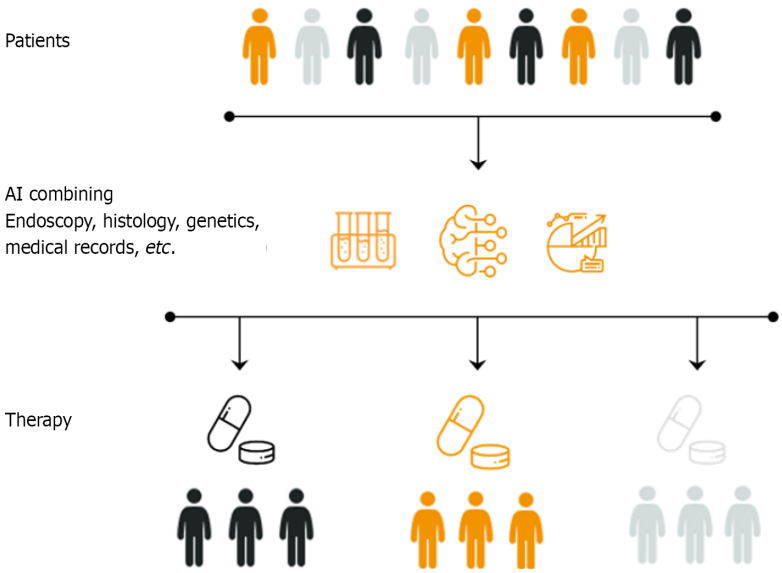
Finally, in order to overcome the fact that conventional DL systems train CNN based on the subjective scoring of images done by clinicians, novel approaches have been developed based on algorithms that only analyze parameters such as pixel color data and vascular pattern recognition. Objective computer-based and operator-independent tools provided a dedicated score that significantly

Table 1 Main studies of artificial intelligence application in the various fields of inflammatory bowel diseases

Ref.	Field	Study features	Main finding
Isakov *et al*[26], 2017	IBD genetics	ML model to assess 16390 genes in IBD and healthy patients	Identified 347 IBD-risk genes (67 newly identified)
Cheng *et al*[27], 2019	IBD genetics	Software analysis to assess the genetics of 32713 IBD patients	Identified several genes potentially involved in UC; identification of 11 common Gene Ontology terms for UC
Yuan *et al*[28], 2017	IBD genetics	Software analysis to assess 12754 genes in IBD and healthy patients	Identified 41 genes closely associated with IBD
Mihajlović *et al*[30], 2021	IBD and microbiota	ML classification algorithm to identify IBD from 1638 fecal samples	Confirmed strong connection between IBD and specific fecal microbial species
Manandhar *et al*[31], 2021	IBD and microbiota	ML model analysis of fecal microbiota from 729 IBD patients and 700 healthy controls	Identified of 117 bacterial taxa with a potential role in diagnostic screening of IBD
Mossotto *et al*[32], 2017	IBD diagnosis	ML model to assess 287 pediatric patients with IBD	Accuracy of 83.3% of the combined endoscopy-histology ML model in the classification of pediatric IBD patients
Quénéhervé *et al*[33], 2019	IBD diagnosis	AI analysis of CLE images from 50 IBD patients and 9 healthy controls	AI analysis had 100% sensitivity and specificity for IBD diagnosis, 92% sensitivity and 91% specificity of IBD differential diagnosis
Ananthakrishnan *et al*[9], 2013	IBD diagnosis and data collection	NLP model trained and validated on 700 UC patients and 700 CD patients to improve case definition and identification from EMRs	NLP model provided better accuracy (AUC 0.94-0.95) than models using only the International Classification of Diseases 9th revision for IBD case definition and identification
Stidham *et al*[39], 2019	IBD endoscopy	DL model for UC severity trained on 16514 endoscopic images	Similar performance of the DL model and experienced human reviewers in grading UC endoscopic severity
Ozawa *et al*[40], 2019	IBD endoscopy	CNN-based CADe system for UC severity trained on 26304 endoscopic images	CADe system had AUCs of 0.86 and 0.98 in the identification of Mayo score 0 and 0-1, respectively
Maeda *et al*[41], 2021	IBD endoscopy	Endoscopic AI model used in real time on 135 UC patients in clinical remission	Endoscopic applications of real time AI predicted clinical relapse of UC with statistical significance
Gottlieb *et al*[42], 2021	IBD endoscopy	DL algorithm to assess UC severity on 795 full-length endoscopy videos	DL algorithm showed significant inter-rater agreement to human central readers for prediction of UC severity
Yao *et al*[43], 2021	IBD endoscopy	Endoscopic AI model (CNN) to assess UC grading used on 169 endoscopy videos and compared to dual central reader review	AI model approximated the scoring of experienced reviewers for grading of UC endoscopic activity
Byrne *et al*[44], 2021	IBD endoscopy	DL model (CNN) to detect and assess UC activity leveraged on > 375000 frames	DL model resulted in well aligned scoring guidelines and experts' performances
Takenaka *et al*[45], 2020	IBD endoscopy	DL algorithm trained on endoscopic images and biopsy results and tested on 875 UC patients	DL model identified with an accuracy > 90% patients in endoscopic and histologic remission
Maeda *et al*[46], 2019	IBD endoscopy (endocytoscopy)	CADe system to predict persistent histologic phlogosis from endocytoscopy validated on 100 UC patient	CADe system provided a diagnostic accuracy of 91% with perfect reproducibility for identification of persistent histologic inflammation
Bossuyt *et al*[47], 2020	IBD endoscopy	AI algorithm based on pixel color data and pattern recognition from endoscopic images tested on 55 patients	AI algorithm ("red density") provided an objective computer-based assessment of UC disease activity with good correlation with endoscopic and histological scoring systems
Aoki *et al*[51], 2019	IBD endoscopy (VCE)	AI system (CNN) tested on 10440 small bowel images for detection of erosions and ulcers in CD	AI system showed an accuracy of 90.8% for detection of erosions and ulcers
Klang *et al*[52], 2020	IBD endoscopy (VCE)	DL algorithm applied on 17640 VCE images for ulcer detection in CD	DL algorithm provided an accuracy ranging from 95.4% to 96.7% with an AUC of 0.99 for ulcer detection
Klang *et al*[53], 2021	IBD endoscopy (VCE)	DL model applied on 27892 VCE images for identification of intestinal strictures in CD	DL model showed an accuracy of 93.5% in stricture identification and excellent differentiation between strictures and other lesions
Ferreira *et al*[54], 2022	IBD endoscopy (VCE)	DL model trained and validated on 8085 VCE images for detection of erosions and ulcers in CD	DL model provided an accuracy of 92.4% and a precision of 97.1% for lesion detection
Aoki *et al*[55], 2020	VCE	Comparison between standard endoscopist reading and reading after AI model screening of 20 full-length VCE videos	The mean VCE video reading time was significantly shorter after AI model (CNN) screening compared to standard reading
Maeda *et al*[56], 2021	IBD endoscopy	Case report of dysplasia detection by AI	AI system (EndoBRAIN) identified 2 colonic lesions that

	(surveillance)	system in a patient with long standing UC	harbored low-grade dysplasia upon histological examination
Fukunaga *et al*[57], 2021	IBD endoscopy (surveillance)	Case report of dysplasia detection by AI system in a patient with long standing UC	AI system (EndoBRAIN) identified rectal lesions that harbored high-grade dysplasia upon histological examination
Reddy *et al*[72], 2019	IBD prognosis	ML model employed on 82 CD patients' EMRs to predict disease course	ML model predicted inflammation severity with high accuracy (AUC 92.8%) from EMR data
Takenaka *et al*[73], 2022	IBD prognosis	ML model validated on endoscopic images and biopsy results from 875 UC patients to predict disease course	Histologic remission detected by the ML model correlated with a significant reduction in clinical relapse, steroid use, hospitalization, and colectomy
Li *et al*[75], 2021	Response to treatment	ML model employed on 174 CD patients to predict response to infliximab	ML model based on clinical and serological parameters showed an accuracy of 0.85 for prediction of response to infliximab
Waljee *et al*[76], 2018	Response to treatment	AI model employed on 472 CD patients to predict response to vedolizumab	AI model based on clinical and serological parameters was able to identify patients that achieved a corticosteroid-free biologic remission at week 52 of vedolizumab
Waljee *et al*[77], 2018	Response to treatment	ML algorithm employed on 491 UC patients to predict response to vedolizumab	ML algorithm based on clinical and serological parameters was able to identify patients that achieved a corticos-teroid-free biologic remission at week 52 of vedolizumab
Doherty *et al*[78], 2018	Response to treatment	AI model to assess response to treatment with ustekinumab in 306 patients with CD	AI model detected patients in remission based on clinical data and fecal microbiota at week 6 and 22 of ustekinumab

AI: Artificial intelligence; AUC: Area under the curve; CADe: Computer-aided detection; CD: Crohn's disease; CLE: Confocal laser endomicroscopy; CNN: Convolution neural networks; DL: Deep learning; UC: Ulcerative colitis; EMR: Electronic medical record; IBD: Inflammatory bowel disease; ML: Machine learning; NLP: Natural language processing; VCE: Video capsule endoscopy.



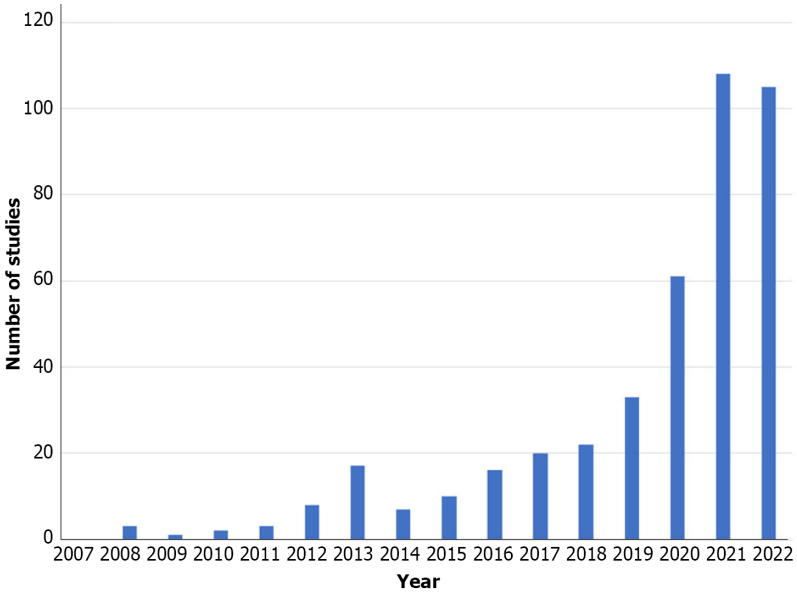
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Figure 1 Application of artificial intelligence in inflammatory bowel diseases for precision medicine. AI: Artificial intelligence.

correlated with both endoscopic and histological scoring systems[47].

Video capsule endoscopy (VCE) represents one of the main modalities of investigating the small bowel in suspect or established CD[48]. The main scoring systems used for disease activity quantification are the Lewis score and the capsule endoscopy CD activity index, which consider parameters such as extension, grading of inflammation, and presence of strictures[49,50].

VCE video review, however, is time-consuming and requires a high level of attention during the observation of thousands of frames. In order to simplify this task, AI has been implemented with the objective of reducing the time needed for image assessment by selecting the most relevant frames or portions of the video. Several CNN models have been developed in order to recognize pathologic findings such as erosions, ulcers, and strictures with very high accuracy[51-54]. Moreover, a study that assessed the review time for VCE showed how the employment of AI systems allowed clinicians to complete the task in a fraction of the time with no differences in overall accuracy[55].



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Figure 2 Clinical studies on the application of artificial intelligence in inflammatory bowel diseases in the last 25 years (as of November 6, 2022).

In addition to recognition of disease activity, AI may find a relevant role in the surveillance of colorectal neoplasia in patients with IBD since a history of long-standing disease is a significant risk factor in developing colorectal cancer[5]. While AI is proving increasingly useful in the detection of colonic neoplasia in the general population with dedicated CADe systems, first attempts at developing specific applications for surveillance in IBD are being made[13]. As of today, successful detection of dysplasia has been described in case reports regarding the application of EndoBRAIN, a CADe system, in endoscopy and endocytoscopy[56,57].

Along with CADe systems, AI may prove to be an effective and safe tool for real-time quality improvement of the examination as well (*i.e.* withdrawal time, checking for blind spots), thereby potentially increasing the adenoma detection rate also in IBD patients[58].

AI IN IBD HISTOLOGY ASSESSMENT

Histological remission is now considered an adjunct target of treatment in UC and arguably the most stringent way to assess remission[59]. Growing evidence shows that persistence of histologic disease activity, even in the absence of macroscopic endoscopic inflammation, is associated with worse clinical outcome and risk of relapse[60]. More than 30 histological scores have been proposed to grade UC histological activity, but their application in clinical practice remains minimal, mainly due to the impracticality of the scores[61,62]. Even when the scores are applied, for example in clinical trials, the interobserver variability is very high, limiting comparison and reproducibility. Indeed, clinical trials increasingly resort to expensive central reading systems so that all biopsies are evaluated by few highly-qualified pathologists to reduce variability. Therefore, AI-based systems to automatically read UC biopsies would be of great help standardizing the assessment and reducing interobserver variability[63].

Trials in this field are ongoing, and initial results are promising. The first attempt to develop a CADe model to assess UC biopsies focused on eosinophils. The system had a good agreement compared to the manual count performed by human pathologists (interclass correlation coefficient = 0.81-0.92) but did not demonstrate an association between eosinophils counted and overall inflammatory activity[64]. More recently, Gui *et al*[65] proposed to simply assess UC activity by taking into consideration the sole presence or absence of neutrophils, the hallmark of active inflammation. They proposed a simplified score, the PICaSSO histologic remission index that was then embedded into a CADe system that was able to distinguish histological activity from remission in biopsies of UC with good accuracy. Further improvements to the same CADe have been recently presented showing that the neutrophil-only assessment by the CADe is largely consistent with mainstay scores such as Robarts and Nancy histological indexes[66].

Other studies on CADe systems for assessment of UC are ongoing and preliminary results are promising[67,68] (Table 2). Further applications of CNN in computational pathology have been studied in order to empower the pathologist's accuracy and efficiency, with models trained on whole slide

Table 2 Artificial intelligence application for histological assessment of ulcerative colitis

Ref.	Study design	Population	Outcome	Results
Vande Casteele *et al*[64], 2022	Cohort study	Colonic biopsies from 88 UC patients with histologically active disease	To assess a DL machine in quantifying eosinophils in colonic biopsies and validate against a pathologist's count	The AI system highly agreed with manual eosinophil count by pathologists (ICC 0.81-0.92)
Peyrin-Biroulet *et al*[67], 2022	Cohort study	200 histological images of UC biopsies	To evaluate an AI algorithm in assessing histological disease activity according to the Nancy index	The CNN model had an excellent agreement with pathologists in the assessment of the Nancy index (ICC 0.84)
Villanacci *et al* [66], 2022	Cohort study	614 biopsies from 307 UC patients	To test a CNN-based CADe system for evaluating HR based on PHRI, Robarts, and Nancy indexes	The CADe system accurately assessed HR (sensitivity 89%, specificity 85% for PHRI) and similar performance for Nancy and Robarts

AI: Artificial intelligence; CADe: Computer-aided detection; CNN: Convolution neural networks; DL: Deep learning; HR: Histological remission; ICC: Interclass correlation coefficient; PHRI: PICaSSO histologic remission index; UC: Ulcerative colitis.

images that were able to effectively support the pathologists by excluding non-diagnostic slides, while retaining optimal sensitivity[69].

AI IN PROGNOSIS AND PREDICTION OF RESPONSE TO TREATMENT

Being able to predict the course of disease with regards to severity and progression is of the utmost importance in IBD patients in order to implement specific management strategies accordingly[70].

Studies have showed that by employing natural language processing and ML algorithms trained on several clinical data from electronic medical records (*i.e.* demographics, laboratory tests, endoscopy reports) it was possible to predict disease severity and surrogate markers of disease flare such as outpatient steroid use or hospitalization[9,71,72].

Furthermore, initial studies on the use of ML to predict the prognosis of patients affected by UC have shown that the endoscopic mucosal healing predicted by a deep neural network model was associated with prognostic features (reduced risks of hospitalization and colectomy) with statistical significance [73].

Lack of response to biological therapies (around 1 out of 3 patients with anti-TNF-alpha therapy) or progressive decline in response over time is an issue of paramount importance in IBD, with a significant economic toll on healthcare costs[74]. For this reason, the possibility to anticipate the likelihood of a patient responding to a specific biological drug prior to its start represents a cost-effective approach to treatment individualization.

ML has been implemented to deal with the complexity of such topics with encouraging results. Through the use of ML on clinical data from trials, random forests were developed to predict the response to biologics such as infliximab, vedolizumab, and ustekinumab, both in CD and in UC with encouraging results[75-78]. The integration and analysis of patients' molecular and clinical features *via* ML algorithms therefore appears to be a promising field with great potential in terms of patient management and optimization of healthcare costs[79].

AI IN PRECLINICAL SETTINGS AND DRUG DISCOVERY

Design and discovery of new drugs are complex processes hampered by high costs and time consumption. Furthermore, especially in recent years, the process of drug discovery has to deal with the increasing wealth and complexity of data from various "omics" (*i.e.* genomics, proteomics)[80].

AI, however, is proving to be a crucial tool in the development of novel drug candidates modernizing this field as well. Among the various implementations of ML and DL in the drug discovery process we can list peptide synthesis, virtual screening (structure-based and ligand-based), prediction of toxicity, drug monitoring and release, quantitative structure-activity relationship, and drug repositioning[81].

In the field of IBD AI has already been employed in drug discovery with encouraging initial results. Computational approaches have been used to identify metabolite-target interactions using the dataset of the IBD cohort from the Human Microbiome Project 2, followed by ML analyses aimed at ranking metabolites according to their importance in IBD and identifying possible human targets through virtual ligand-based screening. Overall, 983 high quality connections between metabolites from the gut microbiota and human proteins possibly relevant to IBD were identified, thus providing multiple novel drug targets for potential immune therapies[82].

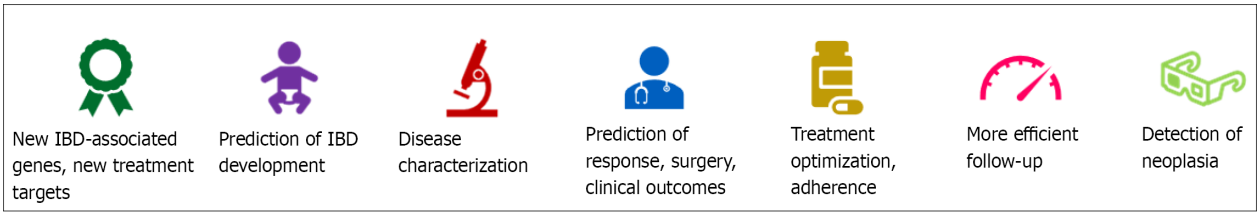
Another relevant application of DL was the construction of a scaffold-based molecular design workflow aimed at developing drug candidates targeted towards the discoidin domain receptor 1. Through a deep generative model, molecular docking and virtual profiling, a high-quality scaffold-based molecular library was established, subsequently leading to the synthesis of a molecular compound that effectively inhibited the expression of proinflammatory cytokines, showing extraordinary kinase selectivity and significant therapeutic protection in *ex vivo* and *in vivo* animal models, respectively[83].

CONCLUSION

Based on the numerous advances in the field of IBD in recent years, it is foreseeable that AI will gain an ever-greater role in the standard patient care, ranging from evaluation of the risk of developing IBD to assistance in the assessment of mucosal activity or detection of dysplasia to support in histopathological reporting, prediction of disease course, and treatment efficacy (Table 1). At the same time, the integration of AI in daily practice will lead to changes in clinical practice itself, getting us closer to the concept of precision medicine and its subsequent improvement in the quality of care and optimization of healthcare costs (Figure 3).

As for research, AI is proving to be an irreplaceable aid in the collection and analysis of large data, while also limiting subjectiveness and interobserver variability, simplifying standardization and providing a feasible alternative to the need of independent central reading in clinical trials. The implementation of AI in everyday practice is expected to improve diagnostic accuracy and reproducibility by allowing for a better standardization of lesion features and classification and by increasing the detection rate of small and subtle mucosal abnormalities or lesions. Moreover, evidence shows how AI may have a role in advanced endoscopic techniques, such as confocal laser endomicroscopy or molecular imaging. Through these advanced application AI will support the clinician by detecting microscopic and even molecular details otherwise invisible, thereby paving the way to the potential incorporation of ultrastructural and molecular endpoints in IBD endoscopy.

Nonetheless, with regard to future applications of AI, there are several issues that will need solving, such as how to provide more transparency of the AI algorithms, how to assess and choose among different models with similar purposes, and how to effectively allocate resources in the plethora of models that will be available, to only list a few[84]. To the best of our knowledge, this is the first review on the roles and the future perspectives of AI in IBD that considers the first clinical applications in a real world setting that are now available from the most recent clinical studies. Nevertheless, the chief limitation is represented by the limited amount of evidence that support our review, which is inevitably due to the scarcity of data currently available for the topic itself from the literature. Undoubtedly further studies are direly needed to build a more robust and comprehensive foundation for future analyses. In brief, while the human component is unlikely to be substituted altogether in future clinical practice and while a few questions still await an answer, AI is an extremely promising means of improvement in patient care and resource optimization.



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Figure 3 Fields of application of artificial intelligence in inflammatory bowel diseases. IBD: Inflammatory bowel disease.

FOOTNOTES

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Country/Territory of origin: Italy

ORCID number: Leonardo Da Rio 0000-0001-5598-5650; Marco Spadaccini 0000-0003-3909-9012; Tommaso Lorenzo Parigi 0000-0002-3398-0231; Roberto Gabbiadini 0000-0002-7277-1053; Arianna Dal Buono 0000-0002-8543-4355; Roberta Maselli 0000000172919110; Alessandro Fugazza 0000-0003-0485-4903; Matteo Colombo 0000-0003-0715-8233; Silvia Carrara 0000-0003-4206-9463; Gianluca Franchellucci 0000-0002-3440-4981; Ludovico Alfarone 0000-0001-9702-4508; Antonio Facciorusso 0000-0002-2107-2156; Cesare Hassan 0000-0001-7167-1459; Alessandro Repici 0000-0002-1621-6450; Alessandro Armuzzi 0000-0003-1572-0118.

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MINIREVIEWS

Role of advanced imaging techniques in the evaluation of oncological therapies in patients with colorectal liver metastases

Martina Caruso, Arnaldo Stanzione, Anna Prinster, Laura Micol Pizzuti, Arturo Brunetti, Simone Maurea, Pier Paolo Mainenti

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Martina Caruso, Arnaldo Stanzione, Arturo Brunetti, Simone Maurea, Department of Advanced Biomedical Sciences, University of Naples "Federico II", Napoli 80131, Italy

Anna Prinster, Laura Micol Pizzuti, Pier Paolo Mainenti, Institute of Biostructures and Bioimaging, National Research Council, Napoli 80131, Italy

Corresponding author: Arnaldo Stanzione, MD, PhD, Postdoc, Department of Advanced Biomedical Sciences, University of Naples "Federico II", Via S. Pansini 5, Napoli 80131, Italy. arnaldo.stanzione@unina.it

Abstract

In patients with colorectal liver metastasis (CRLMs) unsuitable for surgery, oncological treatments, such as chemotherapy and targeted agents, can be performed. Cross-sectional imaging [computed tomography (CT), magnetic resonance imaging (MRI), 18-fluorodexoyglucose positron emission tomography with CT/MRI] evaluates the response of CRLMs to therapy, using post-treatment lesion shrinkage as a qualitative imaging parameter. This point is critical because the risk of toxicity induced by oncological treatments is not always balanced by an effective response to them. Consequently, there is a pressing need to define biomarkers that can predict treatment responses and estimate the likelihood of drug resistance in individual patients. Advanced quantitative imaging (diffusion-weighted imaging, perfusion imaging, molecular imaging) allows the *in vivo* evaluation of specific biological tissue features described as quantitative parameters. Furthermore, radiomics can represent large amounts of numerical and statistical information buried inside cross-sectional images as quantitative parameters. As a result, parametric analysis (PA) translates the numerical data contained in the voxels of each image into quantitative parameters representative of peculiar neoplastic features such as perfusion, structural heterogeneity, cellularity, oxygenation, and glucose consumption. PA could be a potentially useful imaging marker for predicting CRLMs treatment response. This review describes the role of PA applied to cross-sectional imaging in predicting the response to oncological therapies in patients with CRLMs.

Key Words: Colorectal cancer metastases; Prediction response; Computed tomography; Magnetic resonance imaging; Positron emission tomography; Parametric imaging

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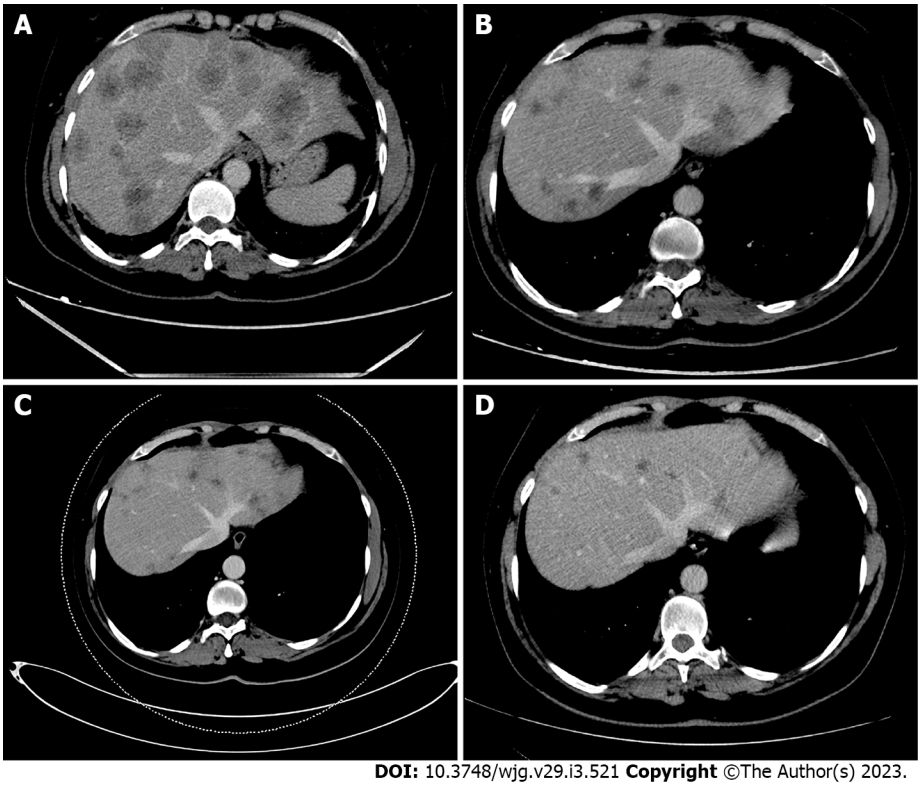
Core Tip: Chemotherapy and targeted agents can be administered to patients with colorectal liver metastasis (CRLM) unsuitable for surgery. The risk of toxicity requires identification of imaging biomarkers that can estimate the likelihood of response and drug resistance before starting therapy. Clinical validation may aid clinicians in tailoring their individual treatment regimens. In this setting, parametric analysis applied to cross-sectional imaging plays a crucial role in evaluating *in vivo* peculiar neoplastic features, such as perfusion, structural heterogeneity, cellularity, oxygenation, and glucose consumption. However, there is no consensus on the most promising imaging quantitative parameter to predict therapy response in CRLMs patients.

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INTRODUCTION

Colorectal cancer is one of the most common cancers worldwide and the fourth leading cause of cancer death[1]. Unfortunately, up to 19% of colorectal cancer patients present with liver metastasis at diagnosis, while up to 13% develop it within the 5-year follow-up[1]. Surgery plays a crucial role in improving the prognosis of colorectal liver metastasis (CRLMs), but only 20% of them are initially suitable for this approach[2]. Hence, systemic chemotherapy is the treatment of choice for the remaining 80% of patients with the aim of rendering metastases resectable and/or prolonging survival[3]. In clinical practice, systemic chemotherapy based on a combination of fluoropyrimidines with oxaliplatin and/or irinotecan is usually associated with targeted agents. The assessment of *KRAS*, *NRAS*, and *BRAF* genes status influences the choice of the most appropriate targeted agents: If they are wild-type, panitumumab or cetuximab, epidermal growth factor receptor (EGFR) antibodies, are preferred, whereas if they are mutated, bevacizumab, a vascular endothelial growth factor (VEGF) antibody, is chosen[4]. In this setting, the cross-sectional imaging evaluation, represented by computed tomography (CT), magnetic resonance imaging (MRI) and 2-[^{18}F]fluoro-2-deoxy-D-glucose positron emission tomography (2-[^{18}F]FDG-PET) associated with CT or MRI (2-[^{18}F]FDG-PET/CT or MRI), is fundamental in the assessment of treatment response based on dimensional evaluation of tumour burden in consecutive scans through the application of standardised criteria, known as "Response Evaluation Criteria in Solid Tumours (RECIST, Figure 1)"[5-7]. These criteria have been very useful in the assessment of treatment response to cytotoxic chemotherapeutic agents, but the introduction of targeted agents with predominant cytostatic effects, such as anti-EGFR and anti-VEGF, makes them insufficient for adequate response imaging evaluation. Indeed, solid tumours may respond to these new agents by developing intra-tumoural necrotic areas and/or cystic, fibrotic, or myxoid degeneration, resulting in an overall increased, decreased, or unchanged size. Thus, the assessment of treatment response during follow-up based purely on dimensional evaluation of the tumour burden seems no longer sufficient. Furthermore, targeted agents are more expensive than cytotoxic agents and are burdened by hepatic toxicity (steatosis, hepatitis, sinusoidal obstruction syndrome, and impaired liver function). Considering these issues, identification of imaging biomarkers that can estimate the likelihood of response and drug resistance in individual patients before or immediately after starting therapy is mandatory. This critical point represents not only a clinician request to avoid unnecessary drug toxicity and the starting delay of alternative therapies, potentially more effective, but also an economic requirement to reduce futile health care costs.

The growth of neoplastic tissue is characterised by the activation of several biological processes, such as neoangiogenesis and anarchic cellular proliferation, which determine neoplastic heterogeneity for the coexistence of high cell density, necrotic, hypoxic, haemorrhagic, and necrotic areas. Neoplastic cells are characterised by increased metabolism and glucose consumption. Currently, these biological neoplastic processes as well as the neoplastic heterogeneity can be analysed *in vivo* applying several post-processing imaging analyses to different cross-sectional imaging techniques, such as diffusion-weighted imaging (DWI) on MRI, perfusion imaging on CT and MRI, and molecular imaging on 2-[^{18}F]FDG-PET/CT and MRI. The *in vivo* structural, functional, and molecular information obtained from imaging is expressed through parametric parameters, which represent potentially useful biomarkers in clinical practice. Parametric analysis (PA) allows the extraction of numerical data contained in the voxels of each image and converts the extracted numerical data into quantitative parametric maps, which are



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Figure 1 Contrast enhanced computed tomography images from a patient with colon cancer. A: Baseline computed tomography (CT) demonstrates the presence of multiple liver metastases; B: After four cycles of combined chemotherapy (folinic acid + fluorouracil + irinotecan + cetuximab) the CT scan shows a reduction in both size and number of liver metastases, which was classified as a partial response with Response Evaluation Criteria in Solid Tumours criteria; C and D: The partial response was then confirmed after (C) 8 and (D) 12 chemotherapy cycles.

representative of peculiar neoplastic features, such as perfusion, structural heterogeneity, cellularity, oxygenation, and glucose consumption, depending on the imaging modalities and techniques used. PA requires the drawing of a region of interest (ROI) or volume of interest that includes the target tissue for analysis. In recent years, PA has been enriched with radiomics, a complex multi-step process that allows the extraction of a huge amount of computational quantitative features from digital medical images, thereby increasing the potential role of cross-sectional imaging in the oncological field. Radiomics has recently emerged as a promising tool for discovering new imaging biomarkers by extracting and analysing numerous quantitative image features representative of tumour heterogeneity and phenotype. Radiomics combines quantitative imaging biomarkers with clinical reports and laboratory test values in statistical models[8].

Finally, the response to chemotherapeutic agents is influenced by their delivery to neoplastic tissues, which is influenced by the tumour microenvironment and cellular characteristics, such as uptake, retention, metabolic activation, and catabolism of drugs, as well as genetic factors such as DNA repair mechanisms[9]. The *in vivo* knowledge of peculiar neoplastic features through cross-sectional imaging may provide imaging biomarkers aiding in the prediction of treatment response and drug resistance. To date, several researchers have investigated the role of quantitative imaging parameters in the pre-treatment response prediction of CRLMs patients using MRI, CT, and 2-[^{18}F]FDG-PET/CT or MRI[10, 11], but there is no clear consensus about which is the most promising imaging technique as well as the most promising quantitative imaging parameter. Therefore, this review aimed to describe the role of PA in predicting the response to oncological therapies in patients with CRLMs.

PA BASED ON MRI DIFFUSION TECHNIQUE

DWI with apparent diffusion coefficient maps

DWI is a functional MRI technique that measures the Brownian motion of water molecules in biological tissues, which is restricted by an increase in cellularity and architectural tissue changes[12]. Consequently, in tumour tissues, the dense cellularity associated with fibrosis, necrosis, neovascularization, and haemorrhages reduces the intercellular space, altering water diffusion properties and restricting Brownian motion. Diffusion-weighted MR images measure the apparent diffusion coefficient (ADC), which is inversely proportional to the cell density, presumably resulting from the tortuosity of

the interstitial space and the consequential limitation of water movement. Tumours with high cellularity tend to present low ADC values on diffusion-weighted MRI because of their high cellularity, characteristically presenting with restriction in these lesions. Therefore, using DWI, it is possible to obtain a parametric ADC map, which is composed of the ADC values calculated for each voxel and represents a quantitative measure of water molecule diffusion expressed as 10^{-3} mm^2/s. ADC is inversely related to tumour cellularity and is strongly affected by molecular viscosity, permeability of the membrane separating the intra- and extracellular compartments, as well as active transport and flow[13]. During treatment, the increase in necrosis, loss of cell membrane integrity, decrease in tumour cellularity, and increase in extracellular spaces determine the increase in water diffusion and, consequently, the increase in ADC values[14]. However, it should be mentioned that a transient decrease in ADC may occur during the first 36–48 h after starting therapy with vascular targeting agents, and the rationale may be the activation of a local immune response, as demonstrated in animal models[15]. Since DWI/ADC provides *in vivo* structural information of tissue composition at any time, and tissue composition influences the treatment response, several authors have investigated the role of this MRI technique in predicting or assessing very early therapy response in patients with CRLMs[16-21]. Most of them are concordant that pre-treatment lower ADC is associated with a better response in CRLMs patients, while a higher ADC is associated with a poorer response[16-18,20]. In particular, Cui *et al*[17] evaluated 11 patients with CRLMs and found that the pre-chemotherapy mean ADC values was significantly lower in responding lesions than those in non-responding lesions ($0.948 \pm 0.147 \times 10^{-3}$ mm^2/s *vs* $1.185 \pm 0.275 \times 10^{-3}$ mm^2/s; $P = 0.003$). Furthermore, Koh *et al*[16] analysed 20 patients with 40 CRLMs and observed that high pre-treatment ADC was predictive of poor response to oxaliplatin- and 5-fluorouracil-base chemotherapy (non-responders: 1.55×10^{-3} mm^2/s; responders: 1.36×10^{-3} mm^2/s; $P < 0.001$). These results were confirmed by Tam *et al*[18] and Fouladi *et al*[20]. The former conducted a study on a larger population composed of 102 patients with CRLMs treated with chemotherapy alone or associated with surgery/radiofrequency ablation (non-responders: 1.40×10^{-3} mm^2/s; responders: 1.16×10^{-3} mm^2/s; $P = 0.024$)[18]. The latter tested the usefulness of baseline 3D ADC to identify the potential responding CRLMs (non-responders: $1332.3 \pm 384.6 \times 10^{-6}$ mm^2/s; responders: $1150.2 \pm 272.9 \times 10^{-6}$ mm^2/s; $P = 0.04$)[20]. Recently, Uutela *et al*[22] investigated the correlation between ADC values at baseline and the RECIST response in a prospective study conducted in 52 patients with CRLM. ADC values below the median of 1.20×10^{-3} mm^2/s at baseline were associated with partial response according to the RECIST criteria 8–12 wk after starting therapy[22].

The biological rationale of these results can be postulated as follows: A higher ADC is observed in necrotic tissues, whereas a lower ADC is observed in viable areas. Necrosis before therapy may indicate a more aggressive phenotype and compromise the delivery of chemotherapeutic drugs; therefore, necrotic areas are usually poorly perfused and tumour cells are exposed to a more hypoxic and acidic environment. These factors reduce the effectiveness of the therapy[23]. However, it should be highlighted that coagulative necrosis does not increase the ADC, which could explain non-responding lesions with lower ADC[16]. In contrast, viable areas are usually well-perfused, facilitating the delivery and retention of anticancer agents.

Currently, there is no consensus regarding the optimal cutoff point ADC value for predicting response to treatment. The most important factors that influence the identification of a generally accepted ADC threshold are several, such as different scanners, methods of acquisition, sequence parameters, and choice of b values. The b-value is a factor that reflects the strength and timing of the gradients used to generate DWI images: The higher the b-value, the stronger the diffusion effect. Koh *et al*[16] identified a mean pre-treatment ADC value of 1.69×10^{-3} mm^2/s for CRLMs that did not respond to chemotherapy with a sensitivity of 60% and a specificity of 100%, whereas Fouladi *et al*[20] proposed the baseline 3D-ADC value as the optimal cut-off point of 1.006×10^{-3} mm^2/s with a sensitivity of 77.4% and a specificity of 91.3%. In this setting, Drewes *et al*[11] conducted a meta-analysis and identified a practical ADC threshold value of 1.2×10^{-3} mm^2/s, below which nearly all responders are situated and no simultaneous overlap with non-responders exists.

Although, according to the aforementioned results, ADC could appear to be a promising predictive biomarker, some studies contradict these previous results[19,21]. Matsushima *et al*[19] did not find a significant difference in ADC values between responders and non-responders to CRLMs treated with bevacizumab. Boraschi *et al*[21] correlated pre-chemotherapy ADC values of 58 CRLMs with histological tumour regression grade (TRG). TRG is a histological descriptive system aimed at grading fibrotic transformation induced in tumours by neoadjuvant therapy. In detail, TRG 1 is represented by fibrosis with no evidence of residual tumour (*i.e.* complete regression), TRG 2 is represented by fibrosis with single cells or rare groups of residual tumour cells, TRG 3 is represented by fibrosis and residual tumour with a dominance of fibrosis, TRG 4 is represented by fibrosis and residual tumour with a dominance of tumour, and TRG 5 is represented by extensive tumour without evidence of regression[24,25]. A non-linear distribution was observed between pre-ADC values and TRG; lower pre-ADC values correlated with TRG 2-3, but an overlap was observed between TRG 1 (complete response) and TRG 4-5 (no response). The heterogeneous structure of liver metastases in terms of cellularity, necrosis, and/or calcification may explain these results.

Finally, the potential role of ADC in the early prediction of therapy response in CRLMs was also evaluated. Cui *et al*[17] observed an increase in ADCs on day 3 or 7 after initiating chemotherapy in responders, suggesting a very early change in tissue composition from a more cellular pre-treatment phenotype to a less cellular or necrotic posttreatment phenotype. Knowledge of therapy effectiveness as soon as possible allows clinicians to prevent overtreatment of non-responder patients, thus avoiding adverse effects.

Intravoxel incoherent motion and diffusion kurtosis imaging

The ADC value is calculated by a mono-exponential relationship between the DWI signal and b-value. Hence, ADC is influenced by tumour heterogeneity and the Gaussian movement of water molecules. The heterogeneity of tumour tissues affects the non-Gaussian diffusion behaviour of water molecules; therefore, new non-mono-exponential diffusion models, such as intravoxel incoherent motion (IVIM) and diffusion kurtosis imaging (DKI), have been proposed to better characterise neoplastic tissues. IVIM assesses both diffusion and microcapillary perfusion changes in tissues by analysing the signal decay curve obtained from multiple b values and provides both diffusion-related parameters, such as the true diffusion coefficient (D) and ADC, and perfusion-related parameters, such as the pseudodiffusion coefficient (D*) and perfusion fraction (f)[26]. DKI estimates and quantifies the skewed distribution of water diffusion based on a probability distribution function[27]. In addition to the diffusion coefficient, DKI extracts the kurtosis value (K) that results from the probability of the diffusion displacement distribution, which is a dimensionless metric. These advanced MRI diffusion techniques have been investigated for predicting the treatment response in patients with CRLM[26,28,29]. Zhang *et al*[29] conducted a prospective study of 40 patients with CRLMs to evaluate the performance of DWI, IVIM, and DKI in predicting therapy response. Their results confirmed the promising role of ADC and suggested the potential role of IVIM and DKI. At baseline, lower ADCs and D on the IVIM parameter map, mean diffusion values, and higher K values on the DKI parameter map correlated with a better response (P = 0.001, < 0.001, = 0.003, = 0.002, respectively), with areas under the curve (AUCs) of 0.845, 0.832, 0.819, and 0.787, respectively. ADC reached the highest AUC (0.845) with a sensitivity of 73.3%, specificity of 84.0%, and cut-off value of 1.107×10^{-3} mm^2/s. In the literature, D is reported to positively correlate with the degree of tumour necrosis as well as ADC; hence, lower D is expression of poor presence of necrosis and better response to chemotherapy[30]. The combination of the aforementioned parameters by logistic regression yielded an AUC of 0.867[29]. Furthermore, Zhou *et al*[28] found that K values were higher in patients with non-responding CRLMs (responders 0.77 ± 0.15 *vs* non-responders 0.90 ± 0.15; P = 0.015), as expression of more complex microstructure, composed of micro-necroses, fibroses, and cystic changes. Finally, Kim *et al*[26] observed a significant change in diffusion parameters of IVIM, such as ADC and D, after the first cycle of therapy in responder patients, whereas perfusion-related IVIM parameters did not change significantly in both groups, suggesting that diffusion-related IVIM parameters are more useful than perfusion-related parameters in differentiating early responders from non-responders, avoiding overtreatment of patients who may not benefit from chemotherapy.

PA BASED ON SPECTROSCOPY

MR spectroscopy (MRS) is an advanced imaging technique that allows for the non-invasive measurement of the levels of some molecules *in vivo*, using the magnetic properties of certain atomic nuclei, such as protons (^{1}H), phosphorus (^{31}P), and carbon-13 (^{13}C)[31]. Therefore, MRS can provide information on tumour pathophysiology and metabolism, potentially influencing treatment planning[32]. Currently, very few studies have investigated the role of MRS in the assessment and prediction of treatment response in patients with CRLMs with poor and discordant results[22,31,33]. In ^{31}P MRS, an increased ratio of phosphomonoesters and nucleoside triphosphate is associated with tumour progression, while it decreases with tumour regression, even in the absence of changes in standard imaging[31]. These results may encourage the use of MRS in monitoring treatment response. Similarly, Kamm *et al*[33] observed a correlation between the maximum levels of 5-FU catabolites on ^{19}F-MRS and the response to treatment in patients with larger CRLMs, suggesting a potential role of MRS in the prediction of therapy response. In contrast, Uutela *et al*[22] did not find a significant association between baseline levels of free choline on ^{1}H-MRS and treatment response according to the RECIST criteria. Therefore, the role of this technique in patients with CRLMs remains to be investigated. Currently, MRS is not yet applied in clinical settings because of technical issues such as the relatively long scan times needed for a good signal-to-noise ratio, as well as the need for additional hardware and expertise in spectral interpretation.

PA BASED ON CONTRAST-ENHANCED CT OR MRI

Neoangiogenesis is induced by the upregulation of vascular growth factors and is required for tumour growth. This leads to the development of a new, altered, and immature microcirculatory network inside

the tumour lesions. The irregular vascular pattern promotes the coexistence of areas of low vascular density and areas of high angiogenic activity; consequently, regions of high cell density and necrotic, haemorrhagic, and myxoid changes are observed. The use of contrast medium and the acquisition of CT or MRI images before and after its intravenous injection allows the assessment of the vascularity of biological tissues *in vivo*, hence the tumours' neoangiogenesis. Tissue contrast enhancement can be evaluated using two different CT or MRI imaging modalities. One is dynamic and is based on repeated high-frequency image acquisition, which allows the assessment of changes in density on CT or signal intensity on MRI over time. The other is based on image acquisition at a fixed time point to obtain at least two or three phases (arterial, portal, and delayed). A broad spectrum of quantitative parameters can be extracted using dynamic acquisition images, which reflect tumour vessel features (perfusion, permeability, and density), extracellular-extravascular space composition, and plasma volume. A summary of the main quantitative parameters used in the assessment of treatment response in patients with CRLM is presented in Table 1. The development of targeting agents with angiogenesis-inhibiting effects, such as bevacizumab, has encouraged studies to examine the correlation between angiogenesis and quantitative imaging parameters. Currently, several potential predictive imaging biomarkers have been identified in different types of cancers, such as renal cell carcinoma, hepatocellular carcinoma, hypopharyngeal carcinoma, and colorectal cancer[10,34-36].

Contrast-enhanced MRI

Few studies have been published regarding the predictive role of MRI quantitative perfusion parameters in patients with CRLMs[37-41]. In particular, Coenegracht *et al*[37] observed in 10 patients with CRLMs a significant difference of K^{ep} values between responders and non-responders (0.09852 *vs* 0.07829; $P < 0.001$); O'Connor *et al*[41] also noticed a high ratio of enhancing tumour voxels to overall tumour voxels in patients with better tumour response. The pathophysiological basis of these results should be as follows: Higher baseline K^{ep} values indicate higher exchange of contrast medium between the blood and the extracellular extravascular space; similarly, a higher exchange of chemotherapy may occur. For this reason and for the presence of an oxygen-rich environment, highly perfused CRLMs at baseline are more likely to respond well to treatment. Furthermore, the role of MRI quantitative perfusion parameters in the prediction of treatment response in patients with CRLMs after the first cycle of a chemotherapy regimen containing bevacizumab has also been investigated. Hirashima *et al*[38] observed a correlation between a higher response and the decrease in K^{trans} ratio (ΔK^{trans}) and K^{ep} ratio (ΔK^{ep}), calculated at baseline and after the first cycle ($P < 0.0001$). De Bruyne *et al*[40] found a correlation between worse response and an increase of at least 40% in K^{trans} after the first cycle of treatment. These results suggest a potential role for quantitative dynamic contrast-enhanced MRI (DCE-MRI) parameters in the early prediction of therapy response and in the assessment of drug resistance[38,40]. On the other hand, Kim *et al*[39] observed discordant results; no significant change in perfusion parameters, such as K^{trans}, K^{ep} and V_e, was found after the first cycle of chemotherapy between responders and non-responders, questioning their role in predicting early therapy response in CRLMs patients.

In clinical practice, gadoxetic acid, a hepatobiliary contrast agent incorporated into hepatocytes by the transporter OATP1B3, is used to better assess CRLMs because of the excellent lesion-to-liver contrast of the hepatobiliary phase (HBP). Murata *et al*[42] investigated the role of gadoxetic acid-enhanced MRI in predicting treatment response in patients with CRLMs. The authors calculated the pre-treatment relative tumour enhancement of the HBP (RTE_{HBP}) in 26 patients with CRLMs using the following formula: RTE values (%) = $[(SI_H - SI_P)/SI_P] \times 100$, where SI_H and SI_P are the signal intensities in the hepatobiliary and pre-contrast phases, respectively. The mean pre-treatment RTE_{HBP} values were significantly higher in responders than in non-responders (37.2% ± 10.9% *vs* 17.9% ± 10.5%; $P = 0.0006$), suggesting a potential association between chemotherapeutic response and OATP1B3 expression. OATP1B3 is an organic anion transporter that is incorporated into hepatocytes, not only in gadoxetic acid, but also in endogenous and exogenous molecules, such as bile acids and chemotherapeutic agents.

Contrast-enhanced CT

CRLMs are generally hypovascular lesions in the portal phase that obtain their blood supply primarily from the hepatic artery; hence, they are arterialised tumours with increased blood flow (BF) and vascular permeability[43]. Based on this assumption, Joo *et al*[44] investigated the haemodynamic features of liver metastases using quantitative colour mapping of the arterial enhancement fraction (AEF) to explore its potential role in the prediction of therapeutic response in patients with CRLMs. The Authors observed a higher mean AEF value of metastatic tumour (58.9 ± 18.8) than that of tumour-adjacent parenchyma (35.5 ± 15.4) and tumour-free parenchyma (26.4 ± 7.5) (all $P < 0.0001$), confirming the arterial vascularisation of liver metastases. Similarly, Kim *et al*[45] extracted some perfusion parameters from perfusion CT of 17 patients with CRLMs and noticed that BF, K^{trans}, and portal liver perfusion were significantly lower in metastatic lesions than in background normal liver parenchyma (41.2 *vs* 50.8, 25.9 *vs* 41.2, 19.3 *vs* 40.9 mL/100 mL/min, respectively), while arterial CRLM perfusion indices were significantly higher than those of hepatic perfusions (28.0 *vs* 22.9 mL/100 mL/min and 57.1% *vs* 26.6%, respectively) ($P < 0.05$). The metastatic blood supply from the hepatic artery and their increased arterial perfusion may be due to the development of a microcirculatory network caused by the neoangiogenesis process. As a consequence, responding lesions of CRLMs patients showed significantly

Table 1 Main quantitative parameters extracted from perfusion computed tomography and magnetic resonance imaging imaging techniques to predict treatment response in patients with colorectal liver metastasis

Parameter name	Parameter definition	Parameter significance
Transfer constant (K^{trans})	Rate of contrast extraction from the blood to the interstitium	It reflects the balance between capillary permeability and BF in a tissue
Tissue interstitial volume (V^e)	Volume of extravascular and extracellular contrast agent in a certain tissue, expressed as a percentage	It is a measure of cell density
Rate contrast (K^{ep})	Rate at which the contrast agent returns from the extravascular-extracellular space to the vascular compartment ($K^{ep} = K^{trans}/V^e$)	It reflects the tissue microcirculation and contrast agent permeability
Regional BF	BF *per* unit volume or mass of tissue (mL of blood/min/100 mL of tissue)	It expresses the rate of the delivery of nutrients and oxygen to a certain tissue

BF: Blood flow.

higher AEF values than that non-responding (65.5 ± 9.6 *vs* 51.3 ± 13.2; $P = 0.005$)[44]. These results are concordant with those of Osawa *et al*[46], who investigated the predictive role of contrast-enhanced CT in patients with CRLM treated with chemotherapy with or without bevacizumab. The authors found a significant correlation between a higher composite endpoint (CE) ratio (ratio of CT value during the arterial phase to unenhanced CT value) at baseline and higher tumour shrinkage after four cycles of chemotherapy associated with bevacizumab ($R^2 = 0.24$, $P = 0.03$), unlike in patients not treated with bevacizumab. Furthermore, among CRLM patients with a high CE ratio at baseline, an increase of 29.6% in the tumour shrinkage rate was observed in those treated with bevacizumab compared with a decrease of 1.46% in those not treated with bevacizumab ($P = 0.03$). Among the CRLMs patients with a low CE ratio at baseline, no significant tumour shrinkage was noted. The rationale for these results is unclear, but we can hypothesise that the presence of higher microvessel density at baseline, evaluated on CT as higher AEF values or higher CE ratios, promotes greater delivery of chemotherapeutic agents. Hence, the assessment of these parameters at baseline could be useful in the prediction of treatment response and drug resistance in patients with CRLMs.

Finally, although Kim *et al*[45] did not find any significant difference in perfusion parameters at baseline between responder and non-responder patients with CRLMs, they observed a significant decrease in BF and K^{trans} after the first cycle of chemotherapy (BF: 28.3% *vs* 5.2%, $P = 0.036$, AUC: 0.806; K^{trans}: 18.7% *vs* 13.0%, $P = 0.027$, AUC: 0.819). The early reduction in perfusion parameters may reflect the inhibiting effect of neo-angiogenesis by anticancer drugs and should encourage clinicians to continue the chosen chemotherapy regimen. Furthermore, the Authors identified a cut-off value for the reduction rate of K^{trans} of 15.0% after the first cycle of chemotherapy, with a sensitivity of 66.7% and specificity of 87.5%[45].

PA BASED ON HYBRID IMAGING

The 2-[^{18}F]FDG-PET/CT and 2-[^{18}F]FDG-PET/MRI are molecular and morphological imaging techniques associated with metabolic and anatomical evaluation of tumour lesions[47,48]. The tracer 2-[^{18}F]FDG, an analogue of glucose, is injected intravenously, transported into cells through membrane glucose transporter proteins, and tends to accumulate in malignant cells because of increased glucose consumption[49]. The uptake of 2-[^{18}F]FDG detected by PET can be quantitatively assessed using different parameters; the main parameters used in the prediction of therapy response in CRLMs patients are shown in Table 2[45,48].

Currently, the quantitative evaluation of tumour metabolism using 2-[^{18}F]FDG-PET integrated with CT or MRI plays a crucial role in the routine management of oncological patients. In patients with colorectal cancer, hybrid imaging aids in the detection of extrahepatic distant metastasis in the evaluation of therapy response, as well as in the follow-up of treated patients with rising serum carcinoembryonic antigen (CEA) levels and no detectable disease on morphological imaging[48]. To date, the role of this hybrid technique in the prediction of treatment response in CRLMs patients remains under investigation. Several studies have shown that standardised maximum uptake value (SUV_{max}) is significantly lower in responders than in non-responders before chemotherapy with or without bevacizumab[10,40,45,50-52]. In detail, Byström *et al*[50] found a mean baseline SUV_{max} of 5.6 in responders and 7.4 in non-responders treated with irinotecan-based chemotherapy ($P = 0.02$). Similarly, De Bruyne *et al*[40] observed a mean baseline SUV_{max} of 3.77 in responders and 7.20 in non-responders treated with FOLFOX/FOLFIRI and bevacizumab followed by surgery ($P = 0.012$). In addition, De Bruyne *et al*[40] did not find any correlation between DCE-MRI parameters, SUV_{max} and anatomical tumour response, suggesting that tumour BF, glucose metabolism, and shrinkage are potentially

Table 2 Main quantitative parameters extracted from the 18-fluorodexoyglucose positron emission tomography used in the prediction of treatment response in patients with colorectal liver metastasis

Parameter name	Parameter definition
SUV_{max}	Uptake value of the pixel with the highest activity inside an ROI divided by the injected dose, which must be corrected for decay and normalised to the patient's weight or body surface
SUV_{mean}	Average of all the uptake values of the pixels within an ROI
MTV	Volume of tumour tissues included in a tridimensional ROI with pathological FDG uptake *via* threshold represented by a settled absolute value or percentage of the SUV_{max} or SUV_{mean}
	It includes both volumetric data and metabolic activity of the tumour
TLG	The product of multiplying SUV_{mean} by MTV
SAM	A marker of total lesion glycolysis, calculated by drawing a volume of interest [VOI(1)] around the tumour and a larger VOI [VOI(2)] around VOI(1)
	SAM = Total SUV VOI1 − (mean BG × volume VOI1)
	Mean BG (background activity) = (total SUV VOI2 − total SUV VOI1)/(volume VOI2 − volume VOI1)

SUV_{max}: Standardised maximum uptake value; SUV_{mean}: Standardised mean uptake value; MTV: Metabolic tumour volume; TLG: Total lesion glycolysis; SAM: Standardised added metabolic activity; FDG: Fluorodexoyglucose; PET: Positron emission tomography; ROI: Region of interest; VOI: Volume of interest.

independent predictors[40]. Mertens *et al*[53] introduced a new metabolic parameter, the standardised added metabolic activity (SAM), which is a marker of total lesion glycolysis that measures the total excess tumoural SUV above the tumour background (Table 2). The authors found a significant difference in both SAM and SUV_{max} at baseline between responders and non-responders (34 *vs* 211, P = 0.002; 3.8 *vs* 7.2, P = 0.021, respectively)[52].

In addition to SUV_{max}, other metabolic parameters have been proposed as predictors of therapy response in patients with CRLMs, such as standardised mean uptake value (SUV_{mean30}), metabolic tumour volume (MTV_{30}) and 30% lesion glycolysis (LG_{30}). SUV_{mean30} was defined as the average value of the SUV of the voxels that showed $SUV_{max} \geq 30\%$. MTV_{30} was defined as the tumour volume segmented *via* the threshold SUV_{mean30} of the lesion. Finally, LG_{30} was obtained by multiplying MTV_{30} by SUV_{mean30}. Kim *et al*[45] observed a higher mean SUV_{mean30} in responder than in non-responder patients with CRLMs on prechemotherapy 2-[^{18}F]FDG-PET/CT (5.2 ± 2.3 *vs* 3.5 ± 1.0, P = 0.046; AUC: 0.792) and a significant difference in the reduction rate of MTV_{30} and LG_{30} between responders and non-responders (18.1% *vs* -5.5%, P = 0.015, AUC: 0.847; 37.9% *vs* 10.7%, P = 0.008, AUC: 0.868, respectively) on 2-[^{18}F]FDG-PET/CT performed 2 or 3 wk after the first cycle of chemotherapy. These results suggest the role of these other 2-[^{18}F]FDG-PET/CT quantitative parameters in the prediction of treatment response in patients with CRLMs. In the same population, Kim *et al*[45] did not observe a significant reduction in the SUV_{max} after the first cycle of therapy between responders and non-responders. A possible explanation is that SUV_{max} is based only on a single pixel and does not consider the entire heterogeneous tumour volume, whereas MTV and LG are volume-based parameters and could be more accurate in the early prediction of treatment response. Similarly, Hendlisz *et al*[51] investigated the potential role of 2-[^{18}F]FDG-PET/CT in the prediction of early response after the first cycle of chemotherapy in patients with CRLMs. To avoid metabolic imaging-based rejection of potentially beneficial therapy, the lowest possible reliable response threshold for FDG uptake changes, the $\Delta SUV_{max} < 15\%$, was applied. Using this threshold, the predictive performance of the metabolic assessment for RECIST response showed a sensitivity of 100%, specificity of 57%, positive predictive value of 43%, and negative predictive value of 100%.

Finally, in the assessment of early response to chemotherapy with 2-[^{18}F]FDG-PET, the flare phenomenon should be considered because it might interfere with the measurement of quantitative parameters. It occurs–1–2 wk after the initiation of chemotherapy and consists of a marked increase in 2-[^{18}F]FDG metabolism in lesions that respond later. Hence, it is recommended to avoid the first two weeks after chemotherapy when performing 2-[^{18}F]FDG-PET/CT evaluation[54].

PA BASED ON RADIOMICS

Radiomics represents a multi-step post-processing technique that can be applied to any medical image to convert it into mineable high-dimensional data (radiomics features). The assumption is that biomedical images contain information that reflects tissue heterogeneity and pathophysiology[8]. Radiomics data can be used alone or with other clinical data to build predictive models and decision-

support tools to aid physicians in clinical practice, potentially improving diagnostic, prognostic, and predictive accuracy. Radiomics analyses represent the pursuit of precision medicine to choose the right treatment for the right patient at the right time. To extract the radiomics features, the first step is image acquisition; the second step is image segmentation, which consists of the 2D or 3D delineation of the ROI, represented by the largest cross-section of the tumour, the whole tumour, or tumour sub-regions; and the third step is the radiomics data extraction (Figure 2). Radiomics features are obtained from the drawn ROI using specifically designed formulae conveying different quantitative parameters, such as first-order (based on histogram analysis of the distribution of individual voxel values without concern for spatial distribution) and second- and higher-order statistical descriptors (accounting for pixel intensity spatial distribution)[55]. Second-order statistical descriptors are generally defined as "texture" features, which describe the statistical interrelationship between voxels with similar or dissimilar contrast values, providing a measure of heterogeneity.

The huge amount of radiomics data extracted from medical images can be more easily handled by artificial intelligence (AI) than traditional statistical methods, and machine learning (ML) is a branch of AI focused on algorithms that can be trained for a task they were not specifically programmed to perform[56]. The algorithms are decision support tools and are mainly used for classification problems. In the oncological field, they are applied to detect and characterise tumour lesions as well as to predict and monitor therapy response. A family of ML algorithms of particular interest is represented by neural networks (NN), a complex model composed of nodes (called neurones) that contribute to the creation of deep, multi-layered networks. The use of NNs with such architectures is commonly referred to as deep learning (DL), which autonomously learns the best features for performing data classification[56]. The texture features utilised were a grey-level co-occurrence matrix (GLCM) and grey-level run length (GLRL). Some features were extracted after pre-processing with a wavelet transform. Supervised classification was achieved using ML approaches: Support vector machine (SVM), k-nearest neighbours, and Random Forest. Texture analysis (TA) is a technique that enables the quantification of variations in pixel intensity, including those imperceptible to the human visual system. TA includes the quantification of grey-level patterns, pixel interrelationships, and the spectral properties of an image[57,58].

Few studies have been published on the role of radiomics applied to CT and MRI images to predict therapy response in patients with CRLMs[59-64].

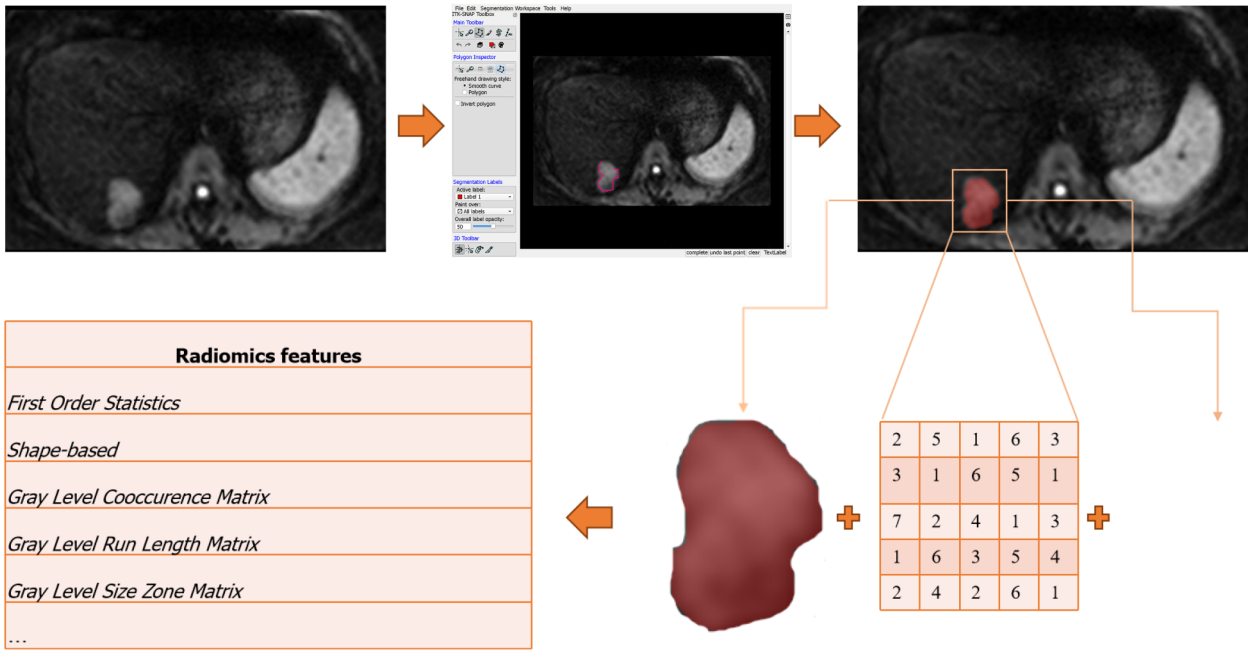
MRI

Zhang *et al*[63] conducted an analysis of T2-weighted images of liver MRI in 26 patients with CRLMs (a total of 193 liver metastases) and extracted five histogram features (mean, variance, skewness, kurtosis, and entropy) and five GLCMs, including angular second moment (ASM), entropy, contrast, correlation, and inverse difference moment. Among the former parameters, only variance was significantly different between CRLMs responders and non-responders ($P < 0.001$), whereas among the latter, all parameters were significantly different ($P \leq 0.001$). Furthermore, when tested using multivariable logistic regression analysis, the association of variance and ASM showed the most potential predictive value for discriminating responders from non-responders with an AUC of 0.814, a sensitivity of 71%, and a specificity of 84.9. These parameters correlate with the complexity and non-uniformity of the image texture, and hence with tumour heterogeneity. According to Zhang *et al*[63], heterogeneous tumours seem to have a more favourable response to therapy. This observation may be related to irregular angiogenesis, greater distribution of tumour blood vessels, and extracellular vascular permeability. Since the effect of drugs relies on their delivery to the tumour site, tumours with greater heterogeneity should theoretically have a better response.

Furthermore, Liang *et al*[62] performed a histogram analysis using ADC maps of 53 patients with CRLMs before starting chemotherapy and observed that the mean, 1st, 10th, 50th, 90th, and 99th percentile values of the ADC maps were significantly lower in responders than in non-responders (P = 0.000-0.002) with AUCs of 0.79, 0.76, 0.76, 0.79, 0.80, and 0.82, respectively. The 99th percentile of ADC showed the highest diagnostic performance for predicting response to chemotherapy, with an AUC of 0.82. These results are concordant with those mentioned above: ADC values included in the 99th percentile are predictive of a good response to chemotherapy, suggesting their association with viable neoplastic tissues, whereas the remaining 1% of ADC values are predictive of insensitivity to chemotherapy, suggesting their association with areas of fluid resulting from necrotic tissues. The authors also investigated histogram-derived CE-MRI parameters extracted from arterial and portal venous phases, such as mean, variance, skewness, kurtosis, and 1st, 10th, 50th, 90th, and 99th percentiles; however, they did not find any significant difference between responders and non-responders in patients with CRLMs. These results may reflect the fact that tumour enhancement is non-specific and influenced by several factors, including BF, capillary permeability, blood volume, and extravascular leakage space[62].

CT

Ahn *et al*[59] conducted a study of 235 patients (145 in the training cohort and 90 in the validation cohort) with CRLMs treated with FOLFOX and FOLFIRI to evaluate several parameters on contrast-enhanced CT, including histogram, volumetric, and morphological features. In multivariate analysis, lower skewness [odds ratio (OR): 6.739, P = 0.003] in 2D analysis, higher mean attenuation (OR: 2.587, P = 0.017), and narrower standard deviation (SD) (OR: 3.163, P = 0.002) in 3D analysis attained statistical



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Figure 2 Schematic diagram showing how radiomics features can be extracted from medical images using a diffusion-weighted imaging image from an magnetic resonance imaging scan of a patient with colorectal liver metastasis as an example. The process begins on the left upper corner with image acquisition, followed by lesion segmentation on a dedicated software leading to a region of interest. The shape of the region of interest as well as the distribution and spatial relation of intensity values of each pixel are computationally analysed to extract radiomics features of different order.

significance for predicting the response of CRLMs to chemotherapy in the training cohort. The lower skewness on 2D images and the narrower SD on 3D images showed good performance in the validation cohorts (AUC: 0.797 and 0.785, respectively). In contrast, Rabe *et al*[64] conducted a CT TA on 29 patients with non-necrotic CRLMs and did not observe a significant correlation between SD and the prediction of response. A possible explanation for these contradicting results could be the exclusion of necrotic lesions in the analysis conducted by Rabe *et al*[64]; indeed, necrosis increases SD. Furthermore, among the several first- and second-order radiomics features extracted by Rabe *et al*[64], eight, such as minimum histogram gradient intensity, skewness, discretised skewness, volume at intensity fraction 10, three GLRL indicators (long run low grey level emphasis, low grey level run emphasis, short run low grey level emphasis), and low grey level count emphasis, were significantly associated with treatment response in univariate analysis. Due to strong correlations within these radiomics features, only two, minimum histogram gradient intensity and long-run grey level emphasis, were included in the multivariate analysis. The AUC of the multivariate model using minimum histogram gradient intensity and long-run grey level emphasis was 0.80, with a sensitivity of 0.73 and a specificity of 0.79 reached with the best threshold of the linear predictor of 0.42. In addition, Ravanelli *et al*[60] investigated the role of contrast-enhanced CT TA in predicting treatment responses to chemotherapy ± bevacizumab in 43 patients with CRLMs. Uniformity was lower in responders than in non-responders ($P < 0.001$) in the bevacizumab-containing chemotherapy group, and in the multivariate analysis, this parameter was independently correlated with radiological CT response at three months (OR: 20, $P = 0.01$). The CT texture parameters were not significantly different between responders and non-responders in the group of patients treated with chemotherapy alone. The correlation between lower uniformity and responders to chemotherapy regimens containing bevacizumab seems to contradict the concept that higher heterogeneity reflects greater aggressiveness. However, it should be highlighted that lesion uniformity is mainly influenced by the presence of angiogenesis, which is triggered and promoted by the upregulation of VEGF, the molecular target of bevacizumab; indeed, the leakage of contrast medium from newly formed and highly permeable tumour microvessels into the extracellular space could account for the small areas of hyper-enhancement that are quantified by the variable uniformity. Hence, the assessment of uniformity should be useful in clinical practice for identifying patients who may benefit from bevacizumab. Low contrast-enhanced CT lesion density was significantly associated with no response in patients with CRLMs treated with chemotherapy with or without bevacizumab ($P = 0.03$ and 0.02, respectively), reflecting poor vascularisation, and thus poor local bioavailability of chemotherapy. Creasy *et al*[61] evaluated, for the first time, the prediction of volumetric response to systemic chemotherapy alone or in association with hepatic artery infusion (HAI), extracting 272 radiomics features from the largest hepatic metastases of 157 colorectal cancer patients. Thirty of the 271 analysed CT radiomics features were selected based on the univariate analysis and used as inputs for

the multivariate regression model. This model was constructed to calculate the percentage of tumour responses. The mean absolute prediction error (MAPE), which represents the mean difference between the predicted response from the model and actual radiographic response, was calculated. MAPE was 16.5% for the training set and 21.5% for the validation set. Furthermore, they conducted a secondary analysis in the validation set stratified by HAI utilisation, demonstrating a MAPE of 19.5% for patients with CRLM treated with HAI and 25.1% for those treated with chemotherapy alone. Since HAI chemotherapy is an expensive treatment with potential complications, predicting the response before starting therapy is very useful for clinicians to choose the most appropriate treatment strategy for each patient.

In the era of personalised medicine, Giannini *et al*[65] developed and validated an ML algorithm to predict the response of individual liver metastases in 24 colorectal cancer patients with a total of 123 lesions, extracting 22 radiomics features on pre-treatment portal CT scans and using an SVM classifier. Their ML algorithm achieved accuracies of 80.9% and 61.5% with sensitivities of 85.7% and 72.7%, and specificities of 66.7% and 47.1%, in the training and test sets, respectively. The prediction of response for each metastasis is crucial in treatment planning because the detection of one or more metastases that will respond differently than others can suggest clinicians to treat them differently[65]. The same group of researchers developed another ML algorithm to predict response in a subgroup of patients with CRLMs and who express HER2 amplification and undergo HER2-targeted therapy[66]. These patients may exhibit a heterogeneous response because some metastases shrink, while others progress[67]. Giannini *et al*[66] extracted 24 radiomics features from a 3D-ROI drawn on baseline portal phase CT and used a Gaussian naïve Bayesian classifier. The radiomics score of individual metastases reached a per-lesion sensitivity of 90% and specificity of 42% in the validation set; thus, the ML algorithm was more accurate in predicting responders than non-responders.

Wei *et al*[68] developed and validated a DL-based radiomics model based on contrast-enhanced CT to predict the response to chemotherapy in patients with CRLMs. The authors compared the diagnostic accuracy of four predictive models based on clinical data and contrast-enhanced CT qualitative features, such as tumour margin, enhanced rim, and target lesion size, DL-based radiomics model, and a combined model. The model that reached the highest AUC was constructed using a combination of CEA level and DL-based fusion radiomics signature (AUC of 0.935 in the training cohort and 0.830 in the validation cohort). Considering that the scanning CT parameters may influence the grey level values and, consequently, the radiomics features, Ahn *et al*[59] compared data acquired with four different CT scanners and did not find any significant differences.

SYNTHESIS OF CURRENT KNOWLEDGE, LIMITS AND FUTURE PERSPECTIVES

The accurate prediction of therapy response in patients with CRLMs is a clinical requirement. In this setting, different imaging techniques such as MRI, CT, and 2-[^{18}F]FDG-PET/CT have been investigated.

PA imaging plays a crucial and promising role. However, evidence is limited, and reproducibility is a major concern. First, most of the studies were retrospective, monocentric, and conducted on small samples, and their results were not validated in the external population. Therefore, prospective, multicentre studies with a larger patient population should be conducted. On the other hand, the identification of a universally accepted cut-off value for each imaging quantitative parameter would be desirable, but it represents a great and ambitious challenge. Indeed, scanners and protocols may influence the value of some quantitative parameters such as ADC and radiomics features.

Regarding contrast-enhanced MRI and CT, perfusion parameters showed the most promising results for predicting therapy response in patients with CRLM. Unfortunately, perfusion techniques have not yet been introduced in routine clinical practice, possibly because of the complexity of the parameter measurements and acquisition protocols. In addition, the quantification of contrast agent concentration is difficult because of the complex relationship between density on CT and signal intensity on MRI and contrast medium concentration, influenced by many factors, such as contrast agent dose, injection rate, time of circulation, and scanner parameters. Finally, the current tumour ROI analysis utilises mean quantitative vascular parameters, which do not accurately reflect the spatial heterogeneity of tumour perfusion.

Regarding hybrid imaging techniques, only a few studies have been published, and they recognise a promising role of 2-[^{18}F]FDG-PET/CT in the prediction of treatment response in patients with CRLM, although this is still under investigation. This technique is not routinely performed in clinical practice, and FDG uptake is influenced by different factors such as tumour grade and histological type.

Recently the emergency of radiomics opens new horizons about the potential role of imaging techniques in predicting tumour response in patients with CRLMs, but currently a lot of issues have to be solved. First, the biological correlation of radiomics features is unclear, and second, imaging acquisition and post-processing may influence the values of radiomics features, hence their reproducibility between different centres. Radiomics represents an ongoing topic of investigation, but its clinical effectiveness remains to be defined.

Hence, considering the great potential of PA in the prediction of therapy response in patients with CRLMs, some issues should be solved. To overcome the lack of reproducibility of quantitative imaging parameters, centre-specific solutions could be hypothesised (*i.e.* each centre could identify its own threshold using the same protocol and the same scanner every time).

Finally, considering that PA is time consuming, the real effect on patient management and outcomes must be defined accurately before introducing it in clinical practice.

CONCLUSION

In an oncological setting, PA applied to cross-sectional imaging allows the extraction of numerical data from neoplastic tissues, which are correlated with morphological, structural, functional, and metabolic features. *In vivo* evaluation of parametric imaging biomarkers can estimate the likelihood of response and drug resistance in individual patients before or immediately after starting chemotherapy and targeted agent therapy. Although the potential role of different imaging quantitative parameters in the prediction of therapy response in patients with CRLMs has been investigated, there is no consensus about which is the most promising parameter; moreover, sometimes the results are controversial. This critical point depends in part on the need for standardisation of the acquisition protocols to obtain data of good quality and reproducibility among different scanners and operators, a well-defined cut-off value, and a clear knowledge of the clinical significance of each imaging quantitative parameter. Therefore, further investigation should be conducted in this field. The identification of a shared quantitative predictive imaging parameter can be of clinical value because it avoids the risk of toxicity in patients who may not benefit from treatment as well as an economic utility to reduce unnecessary healthcare costs.

FOOTNOTES

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Country/Territory of origin: Italy

ORCID number: Arnaldo Stanzione 0000-0002-7905-5789; Arturo Brunetti 0000-0001-7057-3494; Pier Paolo Mainenti 0000-0003-3592-808X.

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ORIGINAL ARTICLE

Retrospective Study

Magnetic resonance imaging-based deep learning model to predict multiple firings in double-stapled colorectal anastomosis

Zheng-Hao Cai, Qun Zhang, Zhan-Wei Fu, Abraham Fingerhut, Jing-Wen Tan, Lu Zang, Feng Dong, Shu-Chun Li, Shi-Lin Wang, Jun-Jun Ma

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Zheng-Hao Cai, Zhan-Wei Fu, Abraham Fingerhut, Lu Zang, Feng Dong, Shu-Chun Li, Jun-Jun Ma, Department of General Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China

Zheng-Hao Cai, Shanghai Minimally Invasive Surgery Center, Shanghai 200025, China

Qun Zhang, Shi-Lin Wang, School of Electronic Information and Electrical Engineering, Shanghai Jiao Tong University, Shanghai 201100, China

Jing-Wen Tan, Department of Radiology, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China

Corresponding author: Jun-Jun Ma, MD, Doctor, Department of General Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, No. 197, Ruijin No. 2 Rd, Shanghai 200025, China. marsnew1997@163.com

Abstract

BACKGROUND

Multiple linear stapler firings during double stapling technique (DST) after laparoscopic low anterior resection (LAR) are associated with an increased risk of anastomotic leakage (AL). However, it is difficult to predict preoperatively the need for multiple linear stapler cartridges during DST anastomosis.

AIM

To develop a deep learning model to predict multiple firings during DST anastomosis based on pelvic magnetic resonance imaging (MRI).

METHODS

We collected 9476 MR images from 328 mid-low rectal cancer patients undergoing LAR with DST anastomosis, which were randomly divided into a training set (n = 260) and testing set (n = 68). Binary logistic regression was adopted to create a clinical model using six factors. The sequence of fast spin-echo T2-weighted MRI of the entire pelvis was segmented and analyzed. Pure-image and clinical-image integrated deep learning models were constructed using the mask region-based convolutional neural network segmentation tool and three-dimensional convolutional networks. Sensitivity, specificity, accuracy, positive predictive value (PPV), and area under the receiver operating characteristic curve (AUC) was calculated

for each model.

RESULTS

The prevalence of ≥ 3 linear stapler cartridges was 17.7% (58/328). The prevalence of AL was statistically significantly higher in patients with ≥ 3 cartridges compared to those with ≤ 2 cartridges (25.0% *vs* 11.8%, P = 0.018). Preoperative carcinoembryonic antigen level > 5 ng/mL (OR = 2.11, 95%CI 1.08-4.12, P = 0.028) and tumor size ≥ 5 cm (OR = 3.57, 95%CI 1.61-7.89, P = 0.002) were recognized as independent risk factors for use of ≥ 3 linear stapler cartridges. Diagnostic performance was better with the integrated model (accuracy = 94.1%, PPV = 87.5%, and AUC = 0.88) compared with the clinical model (accuracy = 86.7%, PPV = 38.9%, and AUC = 0.72) and the image model (accuracy = 91.2%, PPV = 83.3%, and AUC = 0.81).

CONCLUSION

MRI-based deep learning model can predict the use of ≥ 3 linear stapler cartridges during DST anastomosis in laparoscopic LAR surgery. This model might help determine the best anastomosis strategy by avoiding DST when there is a high probability of the need for ≥ 3 linear stapler cartridges.

Key Words: Deep learning; Image-reading artificial intelligence; Magnetic resonance imaging; Predictive model; Double stapling technique; Linear stapler; Rectal cancer; Laparoscopic surgery; Low anterior resection; Anastomotic leakage

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Core Tip: Multiple linear stapler firings during double stapling technique (DST) anastomosis are associated with an increased risk of anastomotic leakage after laparoscopic low anterior resection. This retrospective study developed a deep learning model to predict the use of ≥ 3 linear stapler cartridges during DST anastomosis. With the help of the artificial intelligence to identify and extract information from pelvic magnetic resonance imaging, we developed a clinical-image integrated model with satisfactory accuracy. This model might help preoperatively to determine the anastomosis strategy for rectal cancer patients (suggesting not to perform DST when the risk for ≥ 3 firings is high).

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INTRODUCTION

Anastomotic leakage (AL) is the most common postoperative complication after laparoscopic low anterior resection (LAR) for mid and low rectal cancer[1]. The consequences of AL include higher mortality, need for remedial re-operation, unplanned stoma, delay before adjuvant therapy, and compromised long-term oncological outcomes[2-4]. Although several techniques have been designed to prevent AL[5-9], the prevalence of this complication has hardly improved over the past 20 years[10,11].

Of these techniques, the double stapling technique (DST) has facilitated bowel reconstruction but failed to eliminate AL[2]. During this procedure, the distal margin of the tumor-bearing specimen is transected by one or more linear stapler firings to create the rectal stump. Several publications have identified multiple linear stapler firings as an independent risk factor for AL[1,6,12-16]. Both the Chinese Expert Consensus Statement on the Diagnostic, Prevention and Treatment of the AL for Rectal Cancer (2019) and the United States Food and Drug Administration have suggested limiting the number of stapler firings to two in the DST procedure[17,18]. A recent review of DST suggested that alternative anastomotic techniques to avoid multiple firings on the rectal stump might lower the AL rate[11].

If the number of stapler cartridges used during surgery were predictable before operation, we could predetermine whether DST would be the ideal method for reconstruction. Several studies have reported the association between pelvimetry findings and the technical difficulties (including the use of ≥ 3 linear stapler cartridges) in LAR for mid-low rectal cancer[19-21]. However, previous studies only considered the dimension of pelvic bone landmarks in pelvimetry but ignored mesorectum thickness, tumor size, or tumoral infiltration to nearby organs (prostate, seminal vesicle, uterus). Based on our subjective

experience, we speculated that the narrow (male) pelvis, thick mesorectum, aggressive tumor infiltration, and low transection margin might be associated with the need for ≥ 3 linear stapler cartridges to close the rectal stump. Besides, a simple comparison of one or several measurements of pelvimetry is insufficient to reveal the difficulty of the pelvic procedure. For a lean female patient or a heavy male patient, the same interspinous distance has a vast difference in clinical significance. Furthermore, manual measurement of pelvimetry indicators is time-consuming and labor-intensive.

These shortcomings of existing predictive methods prompted us to design and develop a new model to predict more precisely and effectively the need for ≥ 3 linear stapler firings during DST. Pelvic magnetic resonance imaging (MRI), a routine and first-choice tool for preoperative staging of rectal cancer[22], can capture mesorectal or nearby tissue infiltration characteristics in addition to bony structures. On the other hand, machine learning and deep learning models have been widely applied in health care because of their high ability to predict and make decisions[23]. Owing to the recent technological development[24-25], image-reading artificial intelligence (AI) programs can be used to recognize target features, and then interpret images or provide diagnoses based on these target features[26-30].

In this study, we aimed to create a deep learning pre-warning model for the use of multiple linear stapler cartridges during DST anastomosis by adopting AI to identify, extract and integrate image information from pelvic MRI.

MATERIALS AND METHODS

Patients

We retrospectively analyzed the records of 328 patients who underwent laparoscopic LAR for mid-low rectal cancer at Ruijin Hospital, Shanghai, China, between 2016 and 2021. Clinicopathological data were collected from our prospective institutional database and the study was approved by Ruijin Hospital Ethics Committee (Approval No. 2019-82). Informed consent was waived by the committee because of the retrospective nature of the study. The study was registered at clinicaltrials.gov with the registry number: NCT05498506.

The inclusion criteria were: (1) Rectal carcinoma confirmed by histopathological evaluation; (2) Tumor located in the mid-low rectum (< 10 cm from the anal verge); (3) Performance of DST anastomosis; and (4) Pelvic MRI obtained within 14 d before surgery.

The exclusion criteria were: (1) Other anastomotic techniques (*e.g.*, trans-anal rectal excision); (2) Hartmann's operation or other procedures without anastomosis; (3) Robotic surgery; and (4) The number of linear stapler cartridges was not traceable in the operative report. By using an unbiased random sampling method with a split ratio of 4:1, the patients were divided into a training set (n = 260) and testing set (n = 68).

Surgical procedure

Laparoscopic LAR was performed by one operating team who treated > 200 cases of rectal cancer *per* year. The surgical procedure followed the national guidelines for laparoscopic radical resection of colorectal cancer (2018 edition). Distal rectal transection was performed with an endoscopic linear stapler (Endo-GIA™ Ultra Universal Stapler Reload with Tri-staple™ Technology; Covidien Limited Liability Company, Minneapolis, MN, USA), fired manually through the right lower quadrant 12-mm trocar. The 60-mm purple cartridges containing three different staple heights (3.0 mm, 3.5 mm, and 4.0 mm) were routinely used. However, the 45-mm purple cartridges could be used when the stapler could not be placed perpendicularly to the rectum with the 60-mm cartridges.

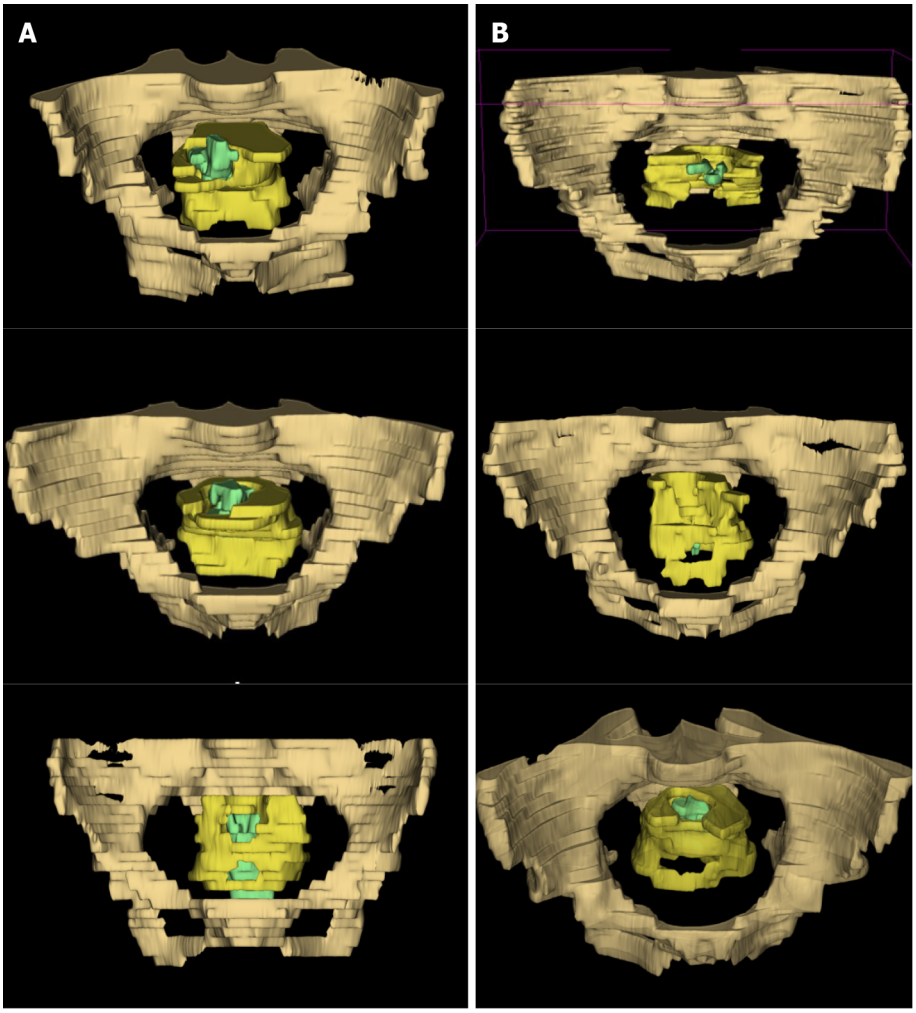
Clinical variables and clinical model

We collected and analyzed baseline characteristics [sex, age, body mass index (BMI)], laboratory analysis [hemoglobin, albumin, carcinoembryonic antigen (CEA)], and tumor features [distance from the anal verge, circumferential resection margin (CRM), tumor size, tumor stage]. For the clinical model, we created a multivariate binary logistic regression model based on clinical variables that might be associated with the number of linear stapler cartridges during surgery: Three binary variables [sex (male, female), CEA level [normal, elevated (> 5 ng/mL)], and CRM (positive, negative)] and three continuous variables (BMI, distance from the anal verge, and tumor size).

MRI protocol and labeling of target region

Pelvic MRI was performed by a Philips INGENIA™ MR scanner with a field strength of 3.0 T and the patient in the supine position. The scanning parameters included: Repetition time = 3565 ms; echo time = 80 ms; layer thickness = 5 mm; image matrix = 312 × 357, field of view = 250 × 340 × 166 mm.

The sequence of fast spin-echo (FSE) T2-weighted MRI with a large field of view with fat suppression obtained in the axial plane of the entire pelvis was retrieved from the Picture Archiving and Communication System for image segmentation. A total of 9476 T2-weighted MR images were collected from the enrolled patients. Fifteen patients in the training set were randomly selected by random



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Figure 1 Examples of three-dimensional model of the target regions. A: Models from patients with the use of ≥ 3 linear stapler cartridges; B: Models from patients with the use of ≤ 2 cartridges. The regions of pelvis, mesorectum, and tumor body were represented by drab, yellow, and green, respectively.

number tables and 367 images from these patients served for manual labeling. A radiological expert with > 15 years of experience in pelvic MRI labeled three target regions (pelvis, mesorectum, and tumor body) on each of the consecutive T2-weighted images. These regions were represented by drab, yellow, and green, respectively (Supplementary Figure 1), using an open annotation tool named Labelme (available at labelme.csail.mit.edu)[31]. Data were transformed into the Common Objects in Context (COCO) dataset format[32].

Segmentation model

Mask region-based convolutional neural network (Mask R-CNN)[24] was used to detect and segment the three target regions (Supplementary Figure 2).

The entire Mask R-CNN network was trained on the training set, and the performance of the testing set was evaluated using the mean Average Precision (mAP). When mAP was > 50, we considered the segmentation model to have performed well[24].

To visualize intuitively the segmentation of the target region, 3D Slicer software (available at www.slicer.org) was adopted to reconstruct a three-dimensional visualization model for each patient (Figure 1).

Deep learning model

A three-dimensional convolutional networks (C3D)-based model was used to generate the probability of multiple linear stapler cartridges after segmentation[25]. We used all the images of one patient as the input whereas the output was the probability of ≥ 3 linear stapler cartridges. When the probability was greater than a preset threshold (set to 0.5 empirically), the sample was judged as positive. We trained the C3D network on the training set for 100 epochs and obtained the final C3D model.

Two deep learning models were used in our study, a pure image model using only T2-weighted MR images segmented by Mask R-CNN and an integrated model using MR images as well as six above-

mentioned clinical variables. The flow chart of the design of these pre-warning models is shown in Figure 2. Our source code is publicly available (https://github.com/suli609/MRI-DST).

Finally, one clinical model and two deep learning models were evaluated on the testing set. A receiver operating characteristic (ROC) curve was plotted for each model. Sensitivity, specificity, accuracy, positive predictive value (PPV), and area under the curve (AUC) were calculated for each curve. AUC > 0.70 indicated an acceptable model.

Statistical analysis

Statistical Package for the Social Sciences (SPSS 13.0, Chicago, IL, USA) was used for statistical analysis. The statistical methods of this study were reviewed by Shuang Wu from China Novartis Institutes for BioMedical Research Co. Ltd. Numerical variables were examined by non-parametric Wilcoxon rank-sum test. Pearson's Chi-Square or Fisher's exact test was adopted to analyze categorical data. Multivariate analysis was performed by binary logistic regression model. The difference was considered statistically significant if two-sided *P* values were < 0.05.

RESULTS

Clinicopathological characteristics of patients

The entire study population included 328 patients, 227 male and 101 female with a median age of 63 (range 24 - 87) years. The prevalence of use of ≥ 3 linear stapler cartridges was 17.7% (58/328). The training set (n = 260) consisted of 48 cases with ≥ 3 cartridges and 212 cases with ≤ 2 cartridges. The testing set (n = 68) consisted of 10 cases with ≥ 3 cartridges and 58 cases with ≤ 2 cartridges.

When clinicopathological characteristics were compared between the patients with ≥ 3 cartridges and those with ≤ 2 cartridges in the training set (Table 1), there was no statistically significant difference between the two groups with respect to sex, age, BMI, diabetes mellitus, preoperative CEA serum level, and the percentage of patients undergoing neoadjuvant chemoradiotherapy. No statistically significant difference was found in the distance from tumor to the anal verge, tumor size, tumor stage, operation time, or insufficient distal resection margin (≤ 5 mm). The incidence of AL was statistically significantly higher in the patients with ≥ 3 cartridges compared to those with ≤ 2 cartridges (P = 0.018).

Univariate and multivariate analysis revealed two independent risk factors for use of ≥ 3 Linear stapler cartridges: Preoperative CEA level > 5 ng/mL (OR = 2.11, 95%CI 1.08-4.12, P = 0.028) and tumor size ≥ 5 cm (OR = 3.57, 95%CI 1.61-7.89, P = 0.002) (Table 2). All these clinicopathological features were comparable between the training set and testing set (Table 3).

Visualization of target regions

Of the three-dimensional reconstruction models presented in Figure 1, those in Figure 1A, 1C, and 1E were models from patients with the use of ≥ 3 linear stapler cartridges while those in Figure 1B, 1D, and 1F were models from patients with the use of ≤ 2 cartridges. Characteristics potentially relevant to the use of ≥ 3 cartridges were narrow pelvis (Figure 1A, drab part), thick mesorectum (Figure 1C, yellow part), and large tumor size with low distal margin (Figure 1E, green part), as can be seen in the models in the left column.

Performance of pre-warning models

The mAP of the segmentation model was 57.2 for the object detection task and 53.7 for the instance segmentation task.

The sensitivity, specificity, and accuracy of the clinical model were 70.0%, 81.0%, and 79.4%, respectively (Youden index = 0.51, PPV = 38.9%). The relevant technical indicators of the image model were as follows: Sensitivity = 50.0%, specificity = 98.3%, accuracy = 91.2%, Youden index = 0.48, and PPV = 83.3%. The integrated model showed the best pre-warning performance: Sensitivity = 70.0%, specificity = 98.3%, accuracy = 94.1%, Youden index = 0.68, and PPV = 87.5%. Finally, the AUC was 0.72, 0.81, and 0.88 for the clinical model, the image model, and the integrated model, respectively (Figure 3).

DISCUSSION

Our deep learning model can predict the probability of using ≥ 3 linear stapler cartridges in the DST anastomosis during laparoscopic LAR surgery. Compared with the clinical model and the pure image model, the integrated model, which combined both the clinical variables and pelvic MR images, had a better Youden index (0.68) and AUC (0.88). Our results suggest that clinical or imaging information alone is insufficient to predict the use of ≥ 3 cartridges during surgery and an MRI-based integrated deep learning model might help determine the best anastomotic strategy for mid-low rectal cancer patients.

Table 1 Clinicopathological characteristics of patients in the training set

Number of linear stapler cartridges	≥ 3	≤ 2	*P* value
	n = 48 (18.5%)	*n* = 212 (81.5%)	
Sex, *n* (%)			0.125
Male	38 (79.2)	144 (67.9)	
Female	10 (20.8)	68 (32.1)	
Age (y), median (quartile)	62 (55-71)	63 (55-68)	0.749
BMI (Kg/m^2), median (quartile)	23.5 (21.1-25.3)	22.9 (21.3-25.1)	0.942
Diabetes mellitus, *n* (%)			0.801
Yes	7 (14.6)	28 (13.2)	
No	41 (85.4)	184 (86.8)	
Hemoglobin (g/L), median (quartile)	136 (124-143)	133 (124-144)	0.540
Albumin (g/L), median (quartile)	39 (36-41)	40 (37-42)	0.015
CEA (ng/mL), median (quartile)	4.27 (2.11-7.08)	3.05 (2.11-5.61)	0.147
nCRT, *n* (%)			0.865
Yes	13 (27.1)	60 (28.3)	
No	35 (72.9)	152 (71.7)	
Distance from anus (cm), median (quartile)	7.2 (5.9-8.4)	7.0 (5.6-8.7)	0.842
CRM evaluated by MRI, *n* (%)			0.103
Positive	16 (33.3)	47 (22.2)	
Negative	32 (66.7)	165 (77.8)	
Operation time (min), median (quartile)	139 (111-180)	143 (116-175)	0.526
Length of cartridges used, *n* (%)			0.113
Only 60 mm	42 (87.5)	200 (94.3)	
45 mm ± 60 mm	6 (12.5)	12 (5.7)	
Anastomotic leakage, *n* (%)			0.018
Yes	12 (25.0)	25 (11.8)	
No	36 (75.0)	187 (88.2)	
Tumor size (cm), median (quartile)	3.7 (3.1-5.1)	3.5 (2.9-4.2)	0.091
T stage, *n* (%)			0.213
T ≤ 2	11 (22.9)	68 (32.1)	
T 3-4	37 (77.1)	144 (67.9)	
N stage, *n* (%)			0.879
N0	25 (52.1)	113 (53.3)	
N+	23 (47.9)	99 (46.7)	
DRM, *n* (%)			0.395
≤ 5 mm	4 (8.3)	27 (12.7)	
> 5 mm	44 (91.7)	185 (87.3)	

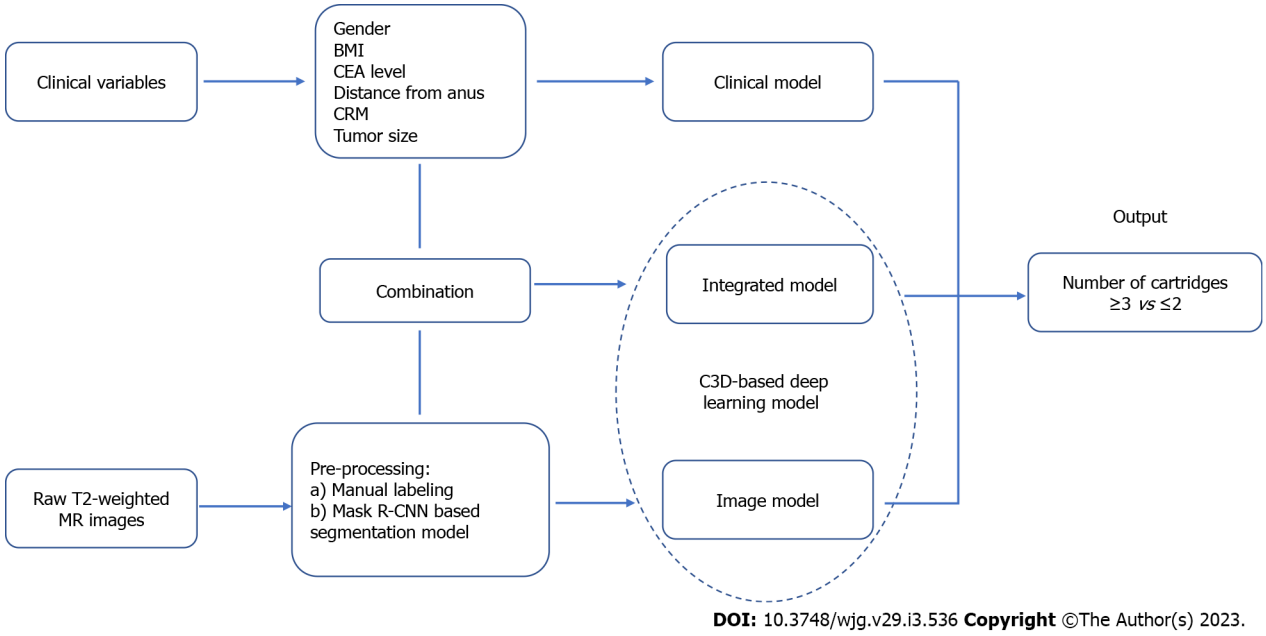
BMI: Body mass index; CEA: Carcinoma embryonic antigen; nCRT: Neoadjuvant chemoradiotherapy; CRM: Circumferential resection margin; MRI: Magnetic resonance imaging; DRM: Distal resection margin.

The safety, feasibility, and oncological outcomes of laparoscopic LAR surgery for mid-low rectal cancer have been confirmed by a series of high-quality randomized controlled trials[33,34]. During laparoscopic LAR, the DST method is considered to be difficult in some patients because the size and

Table 2 Risk factors of ≥ 3 linear staplers in the training set

Factors	Univariate analysis		Multivariate analysis	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Sex (M/F)	0.56 (0.26, 1.18)	0.125	NA	NA
Age (yr) (≥ 70/< 70)	1.60 (0.76, 3.29)	0.205	NA	NA
BMI (Kg/m^2) (≥ 25/ < 25)	1.24 (0.63, 2.44)	0.542	NA	NA
Diabetes mellitus (Y/N)	1.12 (0.46, 2.75)	0.801	NA	NA
Albumin (g/L) (< 35/≥ 35)	2.42 (0.92, 6.37)	0.074	NA	NA
CEA (ng/mL) (> 5/≤ 5)	1.99 (1.04, 3.81)	*0.038*	2.11 (1.08, 4.12)	*0.028*
nCRT (Y/N)	0.94 (0.47, 1.90)	0.865	NA	NA
Distance from anus (cm) (< 5/≥ 5)	0.60 (0.20, 1.79)	0.358	NA	NA
CRM evaluated by MRI (+/-)	1.76 (0.89, 3.47)	0.103	NA	NA
Length of cartridges (mm) (45/60)	0.42 (0.15, 1.18)	0.113	NA	NA
Tumor size (cm) (≥ 5/< 5)	3.38 (1.55, 7.37)	*0.002*	3.57 (1.61, 7.89)	*0.002*

OR: Odds ratio; CI: Confidence interval; NA: Not applicable; BMI: Body mass index; CEA: Carcinoma embryonic antigen; nCRT: Neoadjuvant chemoradiotherapy; CRM: Circumferential resection margin; MRI: Magnetic resonance imaging.



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Figure 2 Flow chart of the design of pre-warning models. BMI: Body mass index; CEA: Carcinoembryonic antigen; CRM: Circumferential resection margin; MR: Magnetic resonance; Mask R-CNN: Mask region-based convolutional neural network.

angle of linear staplers are limited in laparoscopy[14,35]. Consequently, multiple stapler firings are often needed. Two mechanisms might give rise to AL: Either space is left between two adjacent staple lines, or crossing the staple line with another row of staples or crushing the first staple line with the jaws can dislodge, break or deform the staples[6,12,13,18].

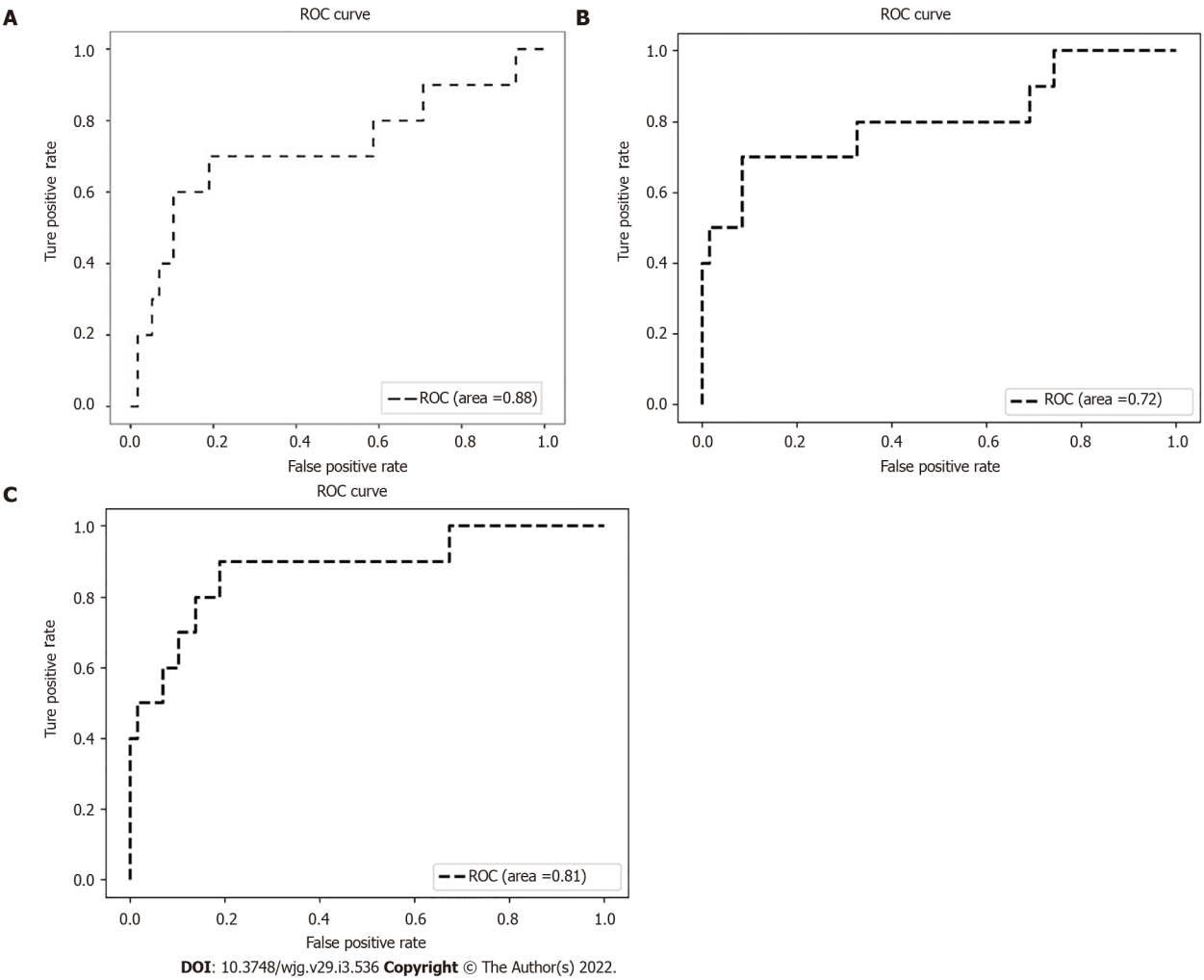
This has prompted surgeons to modify anastomosis techniques, which have been described as follows: Transanal transection of the rectal stump with transanal anastomosis[36,37]; intra-luminal transection of the rectal stump with manual purse-string sutures (*e.g.*, trans-anal total mesorectal excision technique)[37,38]; vertical rectal division using a linear stapler after making an additional skin incision above the pubic symphysis[6]; transverse rectal division using a Contour® stapler during laparoscopic surgery[7]; lateralization of the stump by Nelaton catheter pulling method[8]; side-to-end anastomosis (Baker technique)[9]; trans-anal reinforcement of anastomosis[39]; or removing the "dog ears"/ crossing staple lines[40,41].

Table 3 Comparison between the training set and the testing set

	Testing set	Training set	*P* value
	n = 68	*n* = 260	
Sex, *n* (%)			0.543
Male	45 (66.2)	182 (70.0)	
Female	23 (33.8)	78 (30.0)	
Age (yr), median (quartile)	63 (57-71)	63 (55-68)	0.322
BMI (Kg/m^2), median (quartile)	23.7 (22.0-25.0)	22.9 (21.3-25.1)	0.248
Diabetes mellitus, *n* (%)			0.303
Yes	6 (8.8)	35 (13.5)	
No	62 (91.2)	225 (86.5)	
Albumin (g/L), median (quartile)	39 (36-41)	40 (37-42)	0.111
CEA (ng/mL), *n* (%)		(Missing=5)	0.863
> 5	21 (30.9)	76 (29.8)	
≤ 5	47 (69.1)	179 (70.2)	
nCRT, *n* (%)			0.081
Yes	12 (17.6)	73 (28.1)	
No	56 (82.4)	187 (71.9)	
Distance from anus (cm), median (quartile)	7.1 (5.8-8.7)	7.0 (5.6-8.7)	0.828
CRM evaluated by MRI, *n* (%)			0.051
Positive	9 (13.2)	63 (24.2)	
Negative	59 (86.8)	197 (75.8)	
Tumor size (cm), *n* (%)			0.340
≥ 5	6 (8.8)	34 (13.1)	
< 5	62 (91.2)	226 (86.9)	
Number of linear stapler cartridges, *n* (%)			0.470
≥ 3	10 (14.7)	48 (18.5)	
≤ 2	58 (85.3)	212 (81.5)	
Length of cartridges used, *n* (%)			0.603
Only 60 mm	62 (91.2)	242 (93.1)	
45 mm ± 60 mm	6 (8.8)	18 (6.9)	
Anastomotic leakage, *n* (%)			0.686
Yes	11 (16.2)	37 (14.2)	
No	57 (83.8)	223 (85.8)	

BMI: Body mass index; CEA: Carcinoma embryonic antigen; nCRT: Neoadjuvant chemoradiotherapy; CRM: Circumferential resection margin; MRI: Magnetic resonance imaging.

Thus, if there is a high probability of using ≥ 3 cartridges according to preoperative data, one of these other anastomosis methods might be more suitable than the DST method. Foo *et al*[21] reported a pre-warning model to predict the likelihood of transecting the rectum with ≥ 3 stapler cartridges, which included the following parameters: Sex, pelvic inlet, interspinous distance, intertuberous distance, and tumor height. Two other studies investigated the technical difficulty in LAR surgery with DST anastomosis but they used other indicators, such as operative time, pelvic operative time, blood loss, conversion rate, complications, or specimen quality[42,43]. The factors associated with technical difficulty were BMI, tumor height, interspinous distance, intertubercle distance, pelvic inlet, and pubic tubercle height. The similarity of these studies with ours is that we combined clinical information with



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Figure 3 Receiver operating characteristic curves of the pre-warning models. A: Clinical model; B: Image model; C: Integrated model.

pelvic anatomical factors and the pelvimetry was conducted in pelvic MRI. However, the strengths of our pre-warning model are mainly featured as follows: (1) By using AI-based segmentation of images, the pelvimetry is recognized as a whole instead of isolated measurements; (2) All parameters considered in the above-mentioned clinical models (sex, pelvic measurement, BMI, tumor size/height/stage) were synthesized in our image-reading models. This is why we performed segmentation of three different target regions (bony, fatty, and tumoral) in our study; and (3) This AI-based pre-warning model can shorten the prediction time to 100ms. The only data needed are six clinical factors and the sequence of FSE T2-weighted MR images.

Compared with other segmentation algorithms, such as faster R-CNN, the implementation process of Mask R-CNN is simpler, and the segmentation accuracy is higher. The mAP achieved by our model met the needs of most application scenarios[24]. The actual segmentation effect is close to the target regions manually segmented by radiologists (Supplementary Figure 2). The C3D network structure has good versatility, and the overheads of training the model are small, which is suitable for scenarios with limited training samples[25,44].

Our study had several limitations. First, the small sample size in the testing set lowered the statistical power of our analysis. With this sample size, the statistical difference between the three ROC curves might have been underestimated. Second, the lack of cases made it impossible to validate this model in an external set. Further prospective multi-center studies are needed to verify the validity of this model. Third, deep learning was only conducted on FSE T2-weighted sequences with specific scanning parameters. Further studies could focus on other MRI sequences or contrast-enhanced MRI. Fourth, the number of cartridges was not the only factor involved in AL. The intersection of staple lines[45], the precompression before stapler firings[2], and the distance between the linear staple line and the circular end-to-end anastomosis[35] might also have been implicated in addition to the number of firings. However, we could not include these factors in our analysis because of the retrospective nature of our study. Finally, apart from those factors mentioned above, the number of linear stapler cartridges depended on other factors that were difficult to assess, such as the proper lateralization of the intestinal tube[8] and the precise placement of the trocar through which the linear stapler was fired[2,35]. Thus,

none of our three models achieved 100% accuracy in the testing set. However, the PPV increased to 87.5% in the integrated model compared with 38.9% in the clinical model, indicating that the transabdominal DST method would be unsuitable for positive cases predicted by the integrated model.

CONCLUSION

With the goal of predicting the use of ≥ 3 linear stapler cartridges during DST anastomosis in laparoscopic LAR surgery, our pelvic MRI-based deep learning model might be helpful in the preoperative determination of the best anastomosis strategy for mid-low rectal cancer patients, and, in particular, in avoiding the DST technique when there is a high probability of the need for ≥ 3 linear stapler cartridges. In this setting, another anastomotic technique without staple line crossing should be chosen. Larger studies are needed to validate its clinical value and determine if this strategy can help lower the AL rate.

ARTICLE HIGHLIGHTS

Research background

The need for multiple (≥ 3) linear stapler firings during double stapling technique (DST) is associated with an increased risk of anastomotic leakage (AL) after laparoscopic low anterior resection (LAR).

Research motivation

Current methods using clinical data cannot predict precisely the use of ≥ 3 linear stapler firings before surgery.

Research objectives

This study aimed to develop a pelvic magnetic resonance imaging (MRI)-based deep learning model to predict the multiple firings during DST anastomosis.

Research methods

Clinical data and 9476 MR images from 328 mid-low rectal cancer patients undergoing LAR with DST anastomosis were retrospectively collected. A pure-image model and a clinical-image integrated model were constructed using image-reading deep learning technologies, respectively.

Research results

The clinical-image integrated model showed better predictive performance compared with the clinical model and the pure image model with the highest accuracy (94.1%) and area under the curve (0.88).

Research conclusions

Our deep learning model might help determine the anastomosis strategy for mid-low rectal cancer patients (suggesting not to perform the DST when the risk for ≥ 3 linear stapler firings is high).

Research perspectives

The clinical value of this clinical-image integrated model will be validated in further prospective studies. The incidence of AL is expected to be decreased with this strategy.

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FOOTNOTES

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Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at marsnew1997@163.com. Consent was not obtained but the presented data are anonymized and risk of identification is low.

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Country/Territory of origin: China

ORCID number: Zheng-Hao Cai 0000-0001-5485-3947; Lu Zang 0000-0003-3205-7709; Shu-Chun Li 0000-0001-5719-8917; Jun-Jun Ma 0000-0002-7136-9345.

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SYSTEMATIC REVIEWS

Metabolic dysfunction associated fatty liver disease: The new nomenclature and its impact

Si-Ying Tang, Jian Shiun Tan, Xian-Zheng Pang, Guan-Huei Lee

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Si-Ying Tang, Guan-Huei Lee, Division of Gastroenterology and Hepatology, National University Hospital, Singapore 119228, Singapore

Jian Shiun Tan, Xian-Zheng Pang, Yong Loo Lin School of Medicine, Singapore 117597, Singapore

Corresponding author: Guan-Huei Lee, FRCP, MBBS, MRCP, PhD, Assistant Professor, Attending Doctor, Doctor, Division of Gastroenterology and Hepatology, National University Hospital, 1E, Kent Ridge Road, Singapore 119228, Singapore.
guan_huei_lee@nuhs.edu.sg

Abstract

BACKGROUND

In 2020, an international expert panel proposed a new definition of fatty liver: Metabolic dysfunction-associated fatty liver disease (MAFLD). The MAFLD added the criteria for defining metabolic dysfunctions, which are high-risk factors for liver-related and cardiovascular events. Contrary to the non-alcoholic fatty liver disease (NAFLD) definition, it allows the coexistence of MAFLD and significant alcohol use in the same patient.

AIM

To review the existing data that evaluate the clinical profile and long-term outcome difference between the patients identified as MAFLD and NAFLD.

METHODS

Databases MEDLINE *via* PubMed and EMBASE were searched and relevant publications up to June 28, 2022 were assessed. Studies were included if they involved human participants diagnosed with MAFLD.

RESULTS

A total of 2324 records were reviewed, of which 1575 duplicate citations were removed. Of the 2324 records screened, 207 articles were excluded, and 542 articles were assessed for their eligibility, for which 511 were excluded. The remaining 31 articles were selected for review. MAFLD diagnostic criteria were able to identify more individuals with fatty liver. Studies have shown that patients included using the MAFLD criteria were associated with higher risks of hepatic fibrosis when compared to NAFLD. All-cause mortality, cardiovascular disease-related, and cancer-related mortality were shown to be higher in MAFLD patients. MAFLD patients also had higher baseline metabolic derangement, and risks of

developing obesity, diabetes, and cardiovascular events. Of the 3 subtypes, diabetes mellitus has the strongest association with negative outcomes, followed by metabolic dysfunction and elevated body mass index. Within the subtypes of MAFLD, patients with more metabolic conditions at the time of diagnosis had worse hepatic and liver injury compared to those with a single metabolic condition.

CONCLUSION
MAFLD is a new definition of fatty liver disease that is gaining increasing acceptance. It is based on empirical clinical practice on positive inclusion of metabolic risk factors and recent evidence suggests that it helps to identify patients with higher risk for liver-related as well as cardiovascular events.

Key Words: Hepatic steatosis; Liver fibrosis; Cardiovascular events; Alcohol liver disease; Obesity

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Core Tip: Metabolic dysfunction-associated fatty liver disease (MAFLD) is a new definition of fatty liver disease that is based on positive inclusion of metabolic risk factors. Studies have shown that patients included using the MAFLD criteria were associated with higher risks of hepatic fibrosis and all cause mortality when compared to non-alcoholic fatty liver disease.

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INTRODUCTION

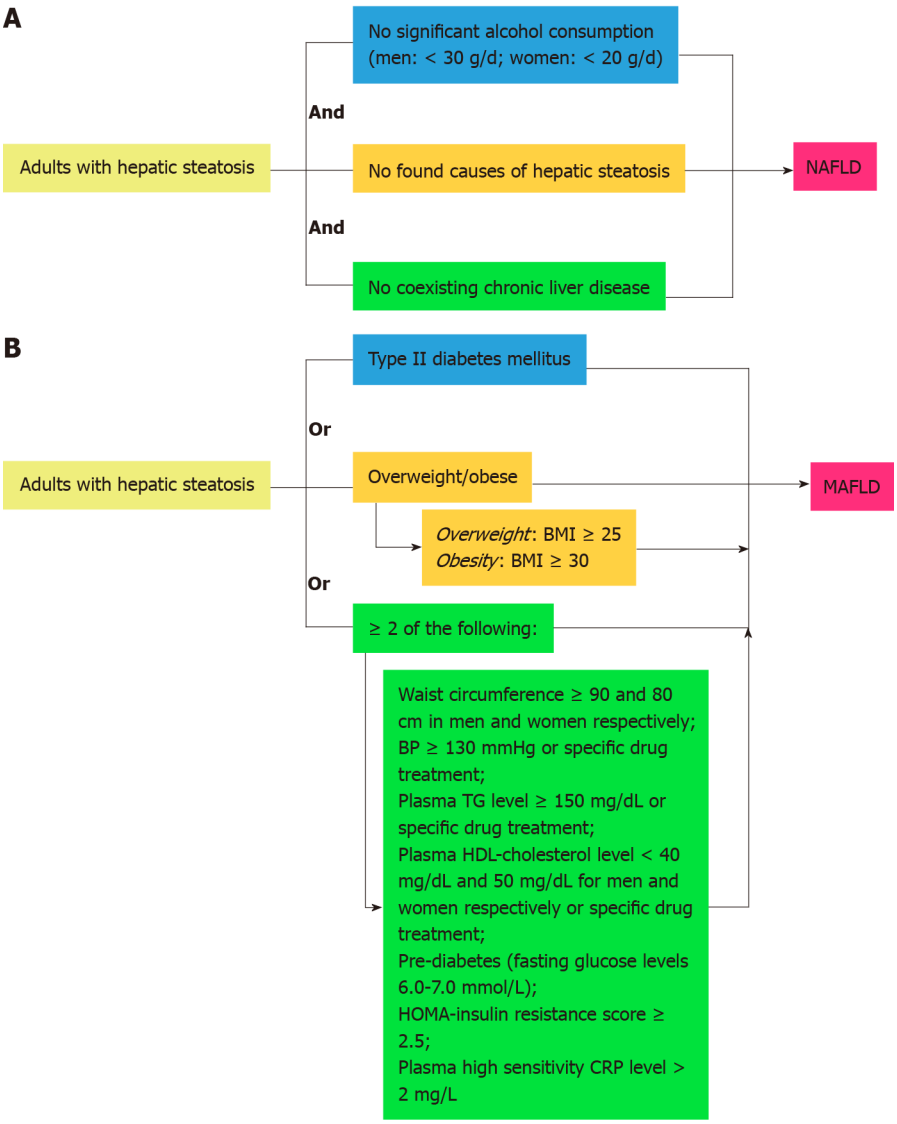
The global prevalence of fatty liver has been rising in recent times, along with metabolic syndrome which are both independently significant contributors to mortality and morbidity worldwide. Since 2020, experts have suggested the change of terminology from non-alcoholic fatty liver disease (NAFLD) to metabolic dysfunction-associated fatty liver disease (MAFLD)[1]. The shift connotes a transition from subtyping patients with hepatic steatosis and no discernible cause of fatty liver, to inclusion criteria characterized by metabolic dysfunction and associated risk factors. NAFLD is an independent disease entity that does not take into account alcohol intake and other causes of pre-existing liver diseases (Figure 1A: Flowchart for the diagnostic criteria of NAFLD).

Metabolic dysfunction in our paper will follow the 1999 World Health Organization definition of metabolic syndrome, which consists of insulin resistance, high fasting glucose, and at least 2 of the following: High-density lipoprotein (HDL)-cholesterol, triglycerides (TG), blood pressure and the presence of obesity. The new proposed MAFLD diagnostic criteria are as follows in Figure 1B (flowchart for the diagnostic criteria for MAFLD): Since the conception of new diagnostic criteria for MAFLD, there have been numerous debates regarding whether this new term should be adopted. There is still a lack of awareness regarding the new terminology and diagnostic criteria amongst many healthcare professionals across the world. This study aims to summarize existing data that evaluate the long-term outcome differences of the change from NAFLD to MAFLD. The study also evaluated the classification of hepatic steatosis by the new MAFLD diagnostic criteria, histopathological classification, as well as risk factors and pathophysiological mechanisms of the new proposed disease entity.

MATERIALS AND METHODS

Eligibility criteria

We included studies ranging from case reports to randomized control trials that have been published till June 28, 2022. We excluded abstracts in this review and have restricted to only studies in English. We excluded studies with insufficient information concerning our outcomes of interest and areas of comparison, *e.g.*, survival, incidence of liver steatosis and severity of fibrosis. A PRISMA checklist was also used to guide the development of the systematic review.



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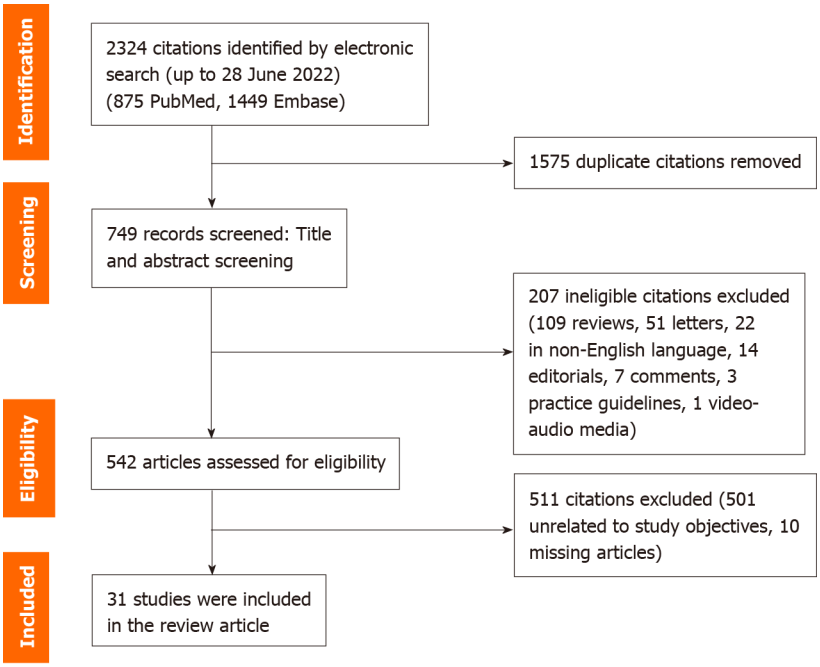
Figure 1 Diagnosis of non-alcoholic fatty liver disease and metabolic dysfunction-associated fatty liver disease[1]. A: Flowchart for the diagnostic criteria for non-alcoholic fatty liver disease; B: Flowchart for the diagnostic criteria for metabolic dysfunction-associated fatty liver disease. NAFLD: Non-alcoholic fatty liver disease; MAFLD: Metabolic dysfunction-associated fatty liver disease; BP: Blood pressure; TG: Triglycerides; HDL: High-density lipoprotein; CRP: C-reactive protein; BMI: Body mass index.

Information sources

A comprehensive systematic search of databases and conference proceedings was conducted to identify all relevant studies up to June 28, 2022. The following electronic databases were searched: MEDLINE *via* PubMed, and EMBASE, with reference to PRISMA guidelines. We used both text words and medical subject heading terms. The literature search strategy was adapted to suit each database. Our search terms included: "Metabolic-Associated Fatty Liver disease" OR "Metabolic dysfunction-associated fatty liver disease" OR "MAFLD *vs* Non-Alcoholic Fatty Liver disease" or "MAFLD *vs* Non-alcoholic Steatohepatitis" OR "Metabolic Associated Steatohepatitis". The methods for data collection and analysis were based on the Cochrane Handbook of Systematic Reviews for Interventions. Where clarification of information in published data was required, corresponding authors were contacted through electronic mail for clarification.

Study selection

Two authors (Tan JS and Pang XZ) independently selected potentially eligible studies using the data management software Rayyan QCRI. The initial screening was based on title and abstract, while final inclusion was based on full texts where available. After reading the titles and abstracts of the identified articles, full-text articles of all citations deemed to meet the inclusion criteria were sought. Duplicates were excluded. Each article was independently inspected to verify that they meet the pre-specified inclusion criteria. The study selection process is summarized in Figure 2 (summary of study selection



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Figure 2 Summary of study selection process.

process). Studies that were included in this systematic review are included in Tables 1 and 2 and Supplementary Tables 1-4. The authors included observational studies reporting the implications of MAFLD *vs* NAFLD.

RESULTS

Search results

A total of 2324 records were reviewed, of which 1575 duplicate citations were removed. Of the 2324 records screened, 207 articles were excluded, and 542 articles were assessed for their eligibility, for which 511 were excluded. The remaining 31 articles that were selected explored various themes, such as the long-term outcome differences of using the MAFLD criteria as compared to the NAFLD criteria, the fibrosis burden in MAFLD as compared to NAFLD, the correlation of MAFLD with other diseases, the histopathological characteristics of MAFLD, as well as risk factors and pathophysiological mechanisms of the new proposed disease entity. Articles that did not compare MAFLD and NAFLD criteria were excluded.

Identification of hepatic steatosis and liver fibrosis

In capturing subjects with hepatic steatosis, the majority of the studies reviewed display a preference for the new MAFLD diagnostic criteria compared with the previous NAFLD, with the new definition being able to identify individuals with dual liver disease etiologies on top of all previously diagnosed NAFLD subjects[2-5]. Results from the Plinio Study also demonstrated that applying the MAFLD criteria reduces the unexplained form of lean NAFLD by identifying the presence of metabolic risk factors in these patients[6]. The Rotterdam Study was also able to identify more individuals with fatty liver disease by applying the MAFLD criteria, where the prevalence of modified MAFLD was higher than NAFLD (34.4% and 29.5%) in their population[7]. MAFLD criteria are also useful in determining the disease severity of patients with diagnosed hepatic steatosis; people with hepatic steatosis who do not fulfil MAFLD criteria are less likely to have significant liver disease as compared to those who are diagnosed with MAFLD (Table 1 and Supplementary Table 1).

In detecting subjects with liver fibrosis, MAFLD criteria also proved superior or concordant with NAFLD in many studies included in this paper[3-5,8],. Results show that the prevalence of significant fibrosis and liver stiffness is considerable in the MAFLD-only group, with marginal differences between the NAFLD-only group and metabolically healthy subjects. One study reported that liver stiffness was higher in MAFLD participants compared to NAFLD participants (7.7 *vs* 6.8 kPa, P = 0.0010)[5]. Compared to NAFLD participants, MAFLD participants also had higher serum liver enzymes (aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transferase), fatty liver index, and fibrosis scores including aminotransferase/platelet ratio index (APRI) and NAFLD fibrosis scores. In MAFLD

Table 1 Overall evaluation of the clinical profile and long-term outcome difference between the patients identified as metabolic dysfunction-associated fatty liver disease and non-alcoholic fatty liver disease (also see Supplementary Tables 1-4 for more details on individual study)

Main outcome	Number of studies	Sample	Conclusion
Hepatic steatosis and fibrosis identification in MAFLD terminology change			
Steatosis and fibrosis	10	38686 subjects	MAFLD definition is able to capture more subjects with fatty liver disease MAFLD group showed either no difference or higher in fibrosis or liver stiffness compared to NAFLD group
Long-term outcome differences in MAFLD terminology change			
All cause mortality risks and cause specific mortality	4	183380 subjects	MAFLD is associated with an increased risk of mortality compared to NAFLD MAFLD mortality is largely contributed by the presence of metabolic disorders
All cause mortality risks	1	12878 subjects	MAFLD and NAFLD share similar all-cause mortality risk MAFLD mortality is hence likely caused by ALD, while NAFLD mortality seems to be caused by metabolic abnormalities
MAFLD and correlation to non-liver diseases			
CVD, ASCVD, cardiovascular events	3	2458240 subjects	The risk of CVD is higher in MAFLD compared to NAFLD MAFLD is superior over NAFLD in predicting ASCVD risk, contributed by the presence of metabolic risk factors
Clinical and histopathological features of MAFLD			
Risk factors, steatosis, advanced fibrosis	9	237679 subjects	T2DM and obesity are significant drivers of MAFLD pathogenesis MAFLD patients had higher BMI, LDL-C and prevalence of T2DM as compared to NAFLD patients Older age, females and menopausal status are risks factors for developing MAFLD

CVD: Cardiovascular disease; ASCVD: Atherosclerotic cardiovascular disease; NAFLD: Non-alcoholic fatty liver disease; MAFLD: Metabolic dysfunction-associated fatty liver disease; T2DM: Type 2 diabetes mellitus; BMI: Body mass index; LDL-C: Low-density lipoprotein cholesterol.

participants with excessive alcohol intake (≥ 30 g/d for males and ≥ 20 g/d for females), it was found that they have a significantly higher APRI score compared to those without excessive alcohol intake[2] (Table 1 and Supplementary Table 1).

However, all the studies reviewed could only provide an estimate of fibrosis and steatosis as the gold-standard technique for diagnosis (liver biopsy) was not done in these large population-based studies. The definition of fibrosis also differed among the studies with one study[7] using liver stiffness ≥ 8.0 kPa as the definition of fibrosis while another[9] defined fibrosis by liver stiffness measure ≥ 9.7 kPa and controlled attenuation parameter ≥ 274 dB/m (Table 1 and Supplementary Table 1).

Park *et al*[10] categorized MAFLD subjects into metabolic health - MAFLD group (≤ 1 risk factor and no diabetes) and metabolic unhealthy MAFLD group (having diabetes and/or ≥ 2 metabolic risk abnormalities) and found that the MH - MAFLD group showed no difference in the prevalence of significant or advanced hepatic fibrosis or carotid artery plaque formation compared with the healthy control group. Between the groups, there were marked differences in comorbidities and hepatic fibrosis burden, suggesting that the MAFLD definition involves an inhomogeneous population at risk of hepatic fibrosis and hence the need for a more elaborate definition (Table 1 and Supplementary Table 1).

There is also a gap in the literature surrounding the application of MAFLD criteria in the pediatric population. Although Ciardullo *et al*[11] managed to find the MAFLD criteria being fulfilled in most of their population (United States adolescents with evidence of hepatic steatosis), it did not affect the prevalence of significant fibrosis and liver stiffness between MAFLD patients and non-MAFLD steatotic patients. This might be due to the inherent chronicity in the progression of hepatic steatosis to liver fibrosis; more time should be granted to investigate the correlation between the new diagnostic criteria and long-term outcomes prospectively in the pediatric population (Table 1 and Supplementary Table 1).

Prediction of long-term outcomes and all-cause mortality

Prospectively, many of the included studies show that individuals with MAFLD demonstrate higher all-

Table 2 Studies included for study of metabolic dysfunction-associated fatty liver disease pathophysiology

Ref.	Type of study	Sample	Main outcomes	Results	Conclusion
Taheri *et al*[29]	Case-control study	968 subjects from Iran	DIS, LIS	Risks of MAFLD (OR): High LIS and DIS > high LIS > high DIS (2.56 *vs* 1.96 *vs* 1.84; $P < 0.001$)	Pro-inflammatory dietary and lifestyle exposures are associated with higher risk of MAFLD regardless of gender. Inflammation may be a primary pathogenic mechanism behind dietary risks of MAFLD development
Mu *et al*[30]	Case-control study	564 subjects from Xinjiang Uygur Autonomous Region, China	SNP	Risks of MAFLD (OR): PNPLA3 rs738409 CC genotype > MBOAT7 rs64173 TT genotype > STAT3 rs74416 AA genotype (1.402 *vs* 1.299 *vs* 0.738; $P < 0.005$)	The CC genotype of PNPLA3 rs738409 and TT genotype of MBOAT7 rs64173 genes are associated with higher risks of MAFLD. The AA genotype of STAT3 rs744166 gene is associated with lower risks of MAFLD. The genes TM6SF2 rs58542926 and GATAD2A rs4808199 show no significant correlation with MAFLD
Panera *et al*[31]	Cohort study-retrospective	1111 subjects from Milan, Italy	Hepatic fibrosis	Associations of KLB rs17618244 variant (OR): Hepatic fibrosis (1.23; $P = 0.04$)	The KLB rs17618244 variant was associated with hepatic fibrosis ($P = 0.04$) but showed no statistical significance in the correlation with steatosis, inflammation and ballooning ($P = 0.37$, 0.12, 0.16 respectively)
Oses *et al*[32]	Cross-sectional study	115 children (8-12 years old)	Fasting blood biochemical parameters, SNP	TG, insulin, HOMA-IR, ALT, AST, GGT, ferritin: MAFLD > non-MAFLD ($P < 0.05$). Percentage of risk of allele carriers: PNPLA3 rs4823173 > PPARG rs1801282 > PPARG rs13081389, HFE rs1800562 (46% *vs* 33% *vs* 21%; $P < 0.05$)	The genetic risk score based on 4 SNPs associated with MAFLD showed limited discriminatory capacity (67% sensitivity and 65% specificity) and did not improve the accuracy of the prediction protocol for MAFLD developed in the study

DIS: Dietary inflammation score; LIS: Lifestyle inflammation score; OR: Odds ratio; NAFLD: Non-alcoholic fatty liver disease; MAFLD: Metabolic dysfunction-associated fatty liver disease; SNP: Single nucleotide polymorphisms; TG: Triglycerides; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transferase.

cause, cardiovascular-related and cancer-related mortality as compared to individuals with NAFLD, or individuals with neither MAFLD or NAFLD[12-15]. A United States study that analyzed 7761 participants with a median follow-up of 23 years, noted that MAFLD patients who do not meet NAFLD criteria have a 1.7-fold higher risk of all-cause mortality, an association not demonstrated in patients with NAFLD or simple hepatic steatosis[15]. Even among MAFLD patients, individuals who meet all 3 criteria of its definition seem to exhibit higher all-cause mortality than those only fulfilling 1 or 2 of the criteria. Individuals who fulfilled all 3 MAFLD criteria had the highest hazard ratio [hazard ratio (HR)] for all-cause mortality risk (HR = 2.05), followed by individuals with metabolic dysfunction and type 2 diabetes mellitus (T2DM) (HR = 1.83), and lastly individuals with only metabolic dysfunction (HR = 1.30)[12] (Table 1 and Supplementary Table 2).

All-cause mortality in MAFLD patients is postulated to be driven by its individual metabolic constituents. Of which, T2DM has the strongest association, followed by metabolic dysfunction and elevated body mass index (BMI)[12-14]. In a United States population study[12], participants with MAFLD were sub-grouped into 1 of the 3 MAFLD criteria and were subsequently analyzed. Interestingly, the overweight (BMI ≥ 25.0 kg/m^2) subgroup was not associated with cancer-related mortality while the metabolic dysregulation subgroup (lean individuals with ≥ 2 metabolic risk factors among non-diabetic participants) was only associated with all-cause mortality, suggesting that T2DM is the most multifaceted cause of mortality in MAFLD patients. A similar study conducted in Kailuan, China showed similar results in that T2DM and metabolic dysfunction have the highest mortality risks (HR = 2.16, 1.79 respectively) among the MAFLD subtypes[14]. A suggested explanation is that on top of proinflammatory, pro-atherogenic and diabetogenic mediators released by livers of patients with NAFLD, the constant exposure to hyperglycaemia and raised concentrations of circulating insulin stimulated cancer progression[12] (Table 1 and Supplementary Table 2).

Age and gender seem to play a role in the mortality risks of MAFLD patients too. Among Kailuan Chinese adults, mortality risks have also been found to be higher in younger adults with MAFLD, with risks declining with age regardless of gender[14]. This association seems to suggest that early-onset metabolic comorbidities are more deleterious in MAFLD patients than when presented at later ages. It is also worth noting that the same study found that obesity has a negative association with mortality risks in older age groups (males above 40 years of age and females above 50 years of age). The non-concordant results could be explained by the obesity paradox, whereby excess adipose tissue could serve as an energy reserve, which could grant a survival advantage in older patients. This might be particularly significant in cancer-related mortality in older MAFLD patients, who are more likely to suffer from malnutrition or poor appetite. A study using LASSO regularisation for variable adjustment found that MAFLD association with cardiovascular-related and cancer-related mortality lost

significance once age, gender and ethnicity were accounted for[13], signifying that age and gender are secondarily important in mortality pathways in MAFLD patients (Table 1 and Supplementary Table 2).

A study of contention points out that the MAFLD definition has failed to capture the impact of metabolic dysfunction on long-term mortality outcomes, attributing the cause of increased all-cause mortality in the MAFLD group to the inclusion of alcoholic liver disease[16] rather than predisposing metabolic derangements. The study demonstrated good concordance between MAFLD and NAFLD groups with similar clinical characteristics except in components of each definition (*e.g.*, alcohol use for MAFLD) and concluded that there was no difference in cumulative all-cause and cause-specific mortality. In another study, individuals with MAFLD, advanced fibrosis was also associated with a higher risk of all-cause mortality [HR = 1.95; 95% confidence interval (CI): 1.46-2.60; $P < 0.001$], while individuals with NAFLD and advanced fibrosis were not significantly associated with all – cause mortality (HR = 1.33; 95%CI: 0.91-1.94; $P = 0.144$)[15]. These findings suggest that MAFLD's strong association with all-cause mortality is independent of known metabolic risk factors, though a point to consider is that mortality risk factors were only retrospectively available for NHANES III data set[15] and not for NHANES 2017-2018 data set reported in the study, which led to fibrosis being used as a surrogate marker for mortality. Contrarily, a study conducted using the Third National Health and Nutrition Examination Survey showed that MAFLD participants had a higher mortality risk regardless of excessive alcohol consumption status over a median follow up of 23.2 years[12] (Table 1 and Supplementary Table 2).

Correlation with cardiovascular and metabolic diseases

NAFLD is tied very closely to cardiovascular diseases (CVD), with CVD being the most important cause of death in NAFLD patients. Hepatic steatosis is independently associated with coronary plaques and both hepatic steatosis and fibrosis are significantly associated with diastolic heart dysfunction. Multiple reports have shown that MAFLD is largely superior to NAFLD in the identification of high-risk patients for atherosclerotic cardiovascular diseases[17-19]. In a retrospective cohort study of 2,452,949 Japanese patients, of which the prevalence of MAFLD was estimated to be 9.7% (n = 237242), the overall prevalence of hypertriglyceridemia, DM and both were 13.6%, 4.3% and 1.1% in non MAFLD patients, compared to 64.1%, 20.6% and 12.9% respectively, in the MAFLD group[17]. The same study also demonstrated that risks of coronary artery disease and CVD were higher in the MAFLD group than in the non-MAFLD group, but the CVD risks were almost the same in NAFLD and non-NAFLD group (HR = 1.02) after adjustments for metabolic syndrome factors, low-density lipoprotein cholesterol (LDL-C), statin use, age, gender, and smoking (Table 1 and Supplementary Table 3).

A single-center cohort study in Japan demonstrated that MAFLD, but not NAFLD, was an independent risk factor for the worsening of atherosclerotic disease[18]. It also identified that the presence of metabolic dysfunction might be the main risk factor for developing cardiovascular disease in MAFLD, instead of alcohol consumption. This suggests that the MAFLD criteria were superior to NAFLD in identifying patients at risk of CVD (Table 1 and Supplementary Table 3).

Patients diagnosed with the MAFLD criteria, but not fulfilling the NAFLD definition, had higher baseline metabolic derangements, except low HDL, compared to patients diagnosed with NAFLD but not fulfilling MAFLD criteria[19]. The same group of patients was also found to have a higher risk of developing general obesity, DM, and cardiovascular events at the end of a 7-year follow (Table 1 and Supplementary Table 3).

Clinical and histopathological characteristics

With the new MAFLD definition gaining traction, many studies have explored methods to characterize the typical patient profile. MAFLD patients tend to be older, have higher BMI, and have more metabolic comorbidities as compared to healthy controls[20]. Unsurprisingly, the presence of metabolic traits meant a higher likelihood of inclusion into the MAFLD population. Compared to NAFLD, the MAFLD population has higher metabolic traits, including high TG, overweight or obesity, glucose intolerance and higher liver enzymes[21]. This result was similar to a study conducted in Fujian, China, where it was found that the MAFLD had higher BMI, LDL-C and T2DM prevalence as compared to NAFLD patients or healthy controls[22] (Table 1 and Supplementary Table 4).

It seems that the number of co-existing metabolic characteristics play an important role in defining the clinical characteristics of MAFLD patients. Patients with two or more metabolic conditions at diagnosis, had a higher grade of hepatic and renal injury compared to those with only one metabolic condition. As the number of concomitant metabolic comorbidities increased, MAFLD patients tended to be older, females, had renal impairment clinically and were more likely to have advanced fibrosis[23] (Table 1 and Supplementary Table 4).

The peak prevalence of MAFLD in the female population is older as compared to the male population [24,25]. This could be due to menopausal factors, where estrogen is postulated to have a protective effect on metabolic disorders. Post-menopausal, lower estrogen levels can lead to fat redistribution and hence result in metabolic disorders such as glucose intolerance, dyslipidemia and MAFLD[24]. It was also found that the odds ratio (OR) of MAFLD was 1.74 times higher for females over 50 years old, than those under 50 years old[26]. On the other hand, older men had a lower prevalence of MAFLD than middle aged men with the prevalence rising rapidly between the age of 18-39, and more slowly after the

age of 40 years with a peak prevalence at 42% in the 50-54 age before declining[25] (Table 1 and Supplementary Table 4).

Among the metabolic subtypes, DM superseded metabolic dysfunction and obesity in prevalence, as well as risks and severity of advanced fibrosis. Among Shanghai Chinese adults, the prevalence of MAFLD and advanced fibrosis was greatest in patients with T2DM, followed by obese and then overweight individuals[20]. In terms of severity, an NHANES III study population found higher fibrosis-4 index (FIB4) scores among MAFLD patients with DM, as compared to metabolic dysfunction and obesity[23]. Similarly, a Taiwanese study found that DM was second to hepatitis B Virus (HBV) infection in its risk of advanced fibrosis in its local MAFLD population, before hypertension or dyslipidemia[27]. More cases of hepatic steatosis and advanced liver fibrosis were found in MAFLD individuals as compared to NAFLD or healthy control groups[21], which might corroborate previous discussions on MAFLD efficacy in identifying liver disease and adverse liver outcomes (Table 1 and Supplementary Table 4).

Different conclusions were made in studies from Fujian, China and Korea. While the former drew similar conclusions in that MAFLD had a higher prevalence of moderate-severe hepatic steatosis than steatotic patients with no metabolic risks, the correlation could not be said the same for the prevalence of advanced fibrosis. However, it is worth considering that many of its participants are selected from a single center with a high proportion of HBV infection and low BMI, which might not adequately capture the relationship between metabolic dysfunction on advanced fibrosis in isolation[22]. In the Korean study, it showed that while metabolic dysfunction did have a positive correlation with risks of liver fibrosis, obesity seemed to be a more contributory factor than DM[28]. An important point worth bringing up is that the mentioned studies used different definitions of advanced fibrosis. While most of the studies collected biopsy-proven liver fibrosis, the definition of advanced differed slightly; the Korean study used defined advanced fibrosis as LSM value ≥ 7.0 kPa[28], the Fujian study as having a score of ≥ 3 on the Scheuer scale[22], the Taiwanese study as stage 3-4 on the NASH CRN fibrosis staging system[27]. To complicate things, some studies used FIB4 scoring as a marker of fibrosis[20,23], which is a measurement done clinically rather than histologically (Table 1 and Supplementary Table 4).

There are few studies comparing the histological profile in NAFLD and MAFLD due to the invasiveness of liver biopsy. One study of 1217 cases did not identify any significant differences in inflammation, advanced fibrosis, and grade of steatosis between MAFLD and NAFLD patients on histology[22]. The same study identified a third group of patients without obesity, T2DM or metabolic dysregulation but with liver steatosis on liver biopsy (non-metabolic related steatosis). Non-metabolic related steatosis patients demonstrated the similar extent of inflammation and degree of fibrosis as MAFLD and NAFLD patients despite being healthier from the metabolic syndrome point of view, hence suggesting that the MAFLD criteria may still miss out on some steatotic patients with significant liver injury (Table 1 and Supplementary Table 4).

Pathophysiology

To date, the exact pathophysiology of MAFLD is not exactly well-understood. Many studies have, however, explored its correlations with genetic variants and modifiable lifestyle practices. Among Iranian adults, higher inflammatory scores secondary to dietary and lifestyle exposures such as smoking and sedentary lifestyles are associated with higher risks of MAFLD. The study suggests that inflammatory mechanisms are intrinsic in the pathophysiologic pathways in MAFLD development and progression[29]. Genetic variants have also been proven to show a link with MAFLD. Among the wide array of variants associated with higher risks of MAFLD include PNPLA3 rs738409 and MBOAT7 rs64173, while variants such as STAT3 rs74416 had been shown to have a protective effect instead. TM6SF2 rs58542926 did not show a significant correlation with MAFLD in the same study[30]. It is worth noting that the three single nucleotide polymorphisms are associated with NAFLD, which implies some degree of shared genetic predisposition to liver disease development. A variant KLB rs17618244 has emerged recently among Italian patients, and results show a predilection for hepatic fibrosis but no correlation to liver steatosis and inflammation[31]. However, the clinical practicality of genetic variant is not yet well-founded; in a pediatric MAFLD population, the genetic risk scores associated with PNPLA3 and PPARG single nucleotide polymorphisms showed little discriminatory value in predicting MAFLD patients[32]. Currently, many studies around MAFLD pathophysiology are limited by small subject groups, and more research should aim toward gaining a deeper and clinically relevant understanding of disease biomechanisms. In comparison, MAFLD shares similar genes as NAFLD, such as PNPLA3, MBOAT7 and TM6SF2[33], although most variants differ between the 2. A meta-analysis found that the PNPLA3 rs738409, also found in MAFLD, showed a positive association with NAFLD, with its G allele being frequently observed in NAFLD individuals (GG *vs* CC OR = 4.01 and GC *vs* CC OR = 1.88)[34] (Table 2).

DISCUSSION

The proposed change of the term from 'NAFLD' to 'MAFLD' aims to better reflect and focus on the

underlying metabolism-related etiology of the disease and not just on the exclusion of alcohol intake or other liver diseases. Our review noted that the MAFLD diagnostic criteria were able to identify more individuals with fatty liver. In terms of advanced fibrosis, the MAFLD criteria were superior or concordant with NAFLD in many studies. All-cause mortality, cardiovascular disease-related and cancer-related mortality were shown to be higher in MAFLD patients. MAFLD patients also had higher baseline metabolic derangement, and risks of developing obesity, diabetes, and cardiovascular events.

Within the subtypes of MAFLD, patients with more metabolic conditions at the time of diagnosis had worse hepatic and liver injury compared to those with a single metabolic condition. This highlights the importance of individualized treatment in MAFLD patients. Non-modifiable risk factors identified for MAFLD include older age, female, post menopause, lower education level, and urban residence and modifiable risk factors include physical activity and BMI. While there are preliminary studies to suggest genetic variants associated with MAFLD, more investigations should be done to explore the mechanism behind them.

From the start, the level of acceptance for the proposal of MAFLD had been varied. So far, the Middle East and North Africa consensus panel and the Latin American Association for the Study of the Liver had endorsed the renaming of NAFLD to MAFLD[35,36]. The Latin American association had also indicated that a change in terminology could increase patients' willingness to openly discuss their disease, as the term "alcohol" leads to stigmatization. The Asian Pacific Association for the Study of the Liver had published clinical practice guidelines for the diagnosis and management of MAFLD[37], noting that dual etiology liver diseases, particularly a combination of MAFLD with viral hepatitis or alcohol, are common in this region. The change in terminology is still being debated in North America and Europe, even though the original expert consensus proposing MAFLD criteria was published in the *Journal of Hepatology*. Recently, it has been proposed that changing the terminology requires a new understanding of the molecular basis of the disease entity and new insights into risk stratification or other important aspects of this liver disease[38]. Central to the debate about the new nomenclature is whether NAFLD is an appropriate name as the term 'non-alcoholic' overemphasizes the absence of alcohol use and underemphasizes the importance of the metabolic risk factors which are the main drivers of disease progression. Further, several investigators have suggested that MAFLD but not NAFLD is associated with increased fibrosis and mortality. The opponents to "MAFLD" raised the concern that there is a lack of a general consensus on the definition of 'metabolic health'. Younossi *et al* [38] reported excess alcohol use was documented in approximately 15% of patients with MAFLD in an NHANES cohort, and contribute to liver-specific mortality for MAFLD (HR = 4.50; 95%CI: 1.89-10.75) but not NAFLD. In the same study, insulin resistance predicted liver-specific mortality in NAFLD (HR = 3.57; 95%CI: 1.35-9.42) but not MAFLD (HR = 0.84; 95%CI: 0.36-1.95). However, as seen, most of the publication to date do report higher fibrosis score.

The major limitation of our study Is, to date, most published studies on MAFLD are retrospective or cross-sectional, with very few prospective studies (which are really "retrospective-prospective", designed before the MAFLD was defined). This is not surprising since the consensus statement was only published in 2020. Second, many large database studies contain data obtained more than 10 years ago. The subjects were unlikely to have been screened comprehensively using the metabolic risk tests as listed in Figure 1B, or received the pharmacotherapies available today. Also, as MAFLD overlaps with NAFLD patients, the use of student *t*-tests and most parametric tests for comparison between the two groups is inappropriate as they are not independent groups. Publishing bias may exist as published studies are mostly positive studies and negative studies may not be reported. Lastly, most of the studies that have been included are over-represented by the Western population, and the generalizability of the results to the rest of the world can be questioned.

CONCLUSION

In conclusion, MAFLD is a new definition of fatty liver disease that is gaining wide acceptance, especially in Asia, Latin America, and Africa. There are still questions in hot debates. The concept is based on empirical clinical practice on positive inclusion of metabolic risk factors and recent evidence suggests that it helps to identify patients with higher risk for liver-related as well as cardiovascular events. MAFLD also consists of three subtypes, each with a unique metabolic dysfunction, which may be useful for the development of new pharmacotherapy. The nomenclature and metabolic risk factor criteria will likely evolve with time. However, the principle of having "positive criteria" for metabolic dysfunction as an etiology for fatty liver disease, independent of alcohol intake, will probably prevail. More high-quality scientific evidence is still required before the widespread acceptance of this new definition.

ARTICLE HIGHLIGHTS

Research background

Metabolic dysfunction-associated fatty liver disease (MAFLD) was proposed in 2020 as the new definition of fatty liver. Compared to nonalcoholic fatty liver disease (NAFLD), MAFLD consists of inclusion criteria characterized by metabolic dysfunction and associated risk factors. There is still a lack of awareness regarding this new MAFLD terminology and its impact on clinical practice.

Research motivation

There have been numerous debates regarding whether the new term MAFLD should be adopted. The definition of MAFLD reflects a shift in the focus from sub typing patients with hepatic steatosis and no discernible cause of fatty liver to the underlying metabolism - related etiology of the disease.

Research objectives

This study summaries existing data that evaluate the long-term outcome differences of the terminology change from NAFLD to MAFLD, classification of hepatic steatosis, histopathological classification, risk factors and pathophysiological mechanisms of the new proposed terminology.

Research methods

A systemic search of database MEDLINE *via* PubMed and EMBASE were conducted to identify relevant studies up to June 28, 2022.

Research results

Of the 2324 records screened, 1575 duplicates were removed, following which 207 articles were excluded and a remaining 542 articles were assessed for eligibility. 511 articles were excluded and a remaining 31 articles were selected for review. Studies show that MAFLD patients were able to identify more patients with fatty liver compared to NAFLD. MAFLD criteria was also superior or concordant in terms of advanced fibrosis. MAFLD is also associated with higher all-cause mortality, cardiovascular disease - related and cancer - related mortality compared to NAFLD patients.

Research conclusions

MAFLD is gaining acceptance as a new definition of fatty liver disease. The nomenclature and definition of MAFLD highlights the metabolic risk factor which are main drivers of disease progression.

Research perspectives

MAFLD consists of 3 subtypes, each with a unique metabolic dysfunction profile that may be useful for development of new pharmacotherapy. However, further understanding is required to determine the molecular basis of MAFLD as a disease entity and new insights into risk stratification.

FOOTNOTES

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Country/Territory of origin: Singapore

ORCID number: Si-Ying Tang 0000-0001-9487-9404; Jian Shiun Tan 0000-0002-0731-7938; Xian-Zheng Pang 0000-0003-4708-4216; Guan-Huei Lee 0000-0002-8652-6403.

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CASE REPORT

Small intestinal angiosarcoma on clinical presentation, diagnosis, management and prognosis: A case report and review of the literature

Xiao-Mei Ma, Bao-Shun Yang, Yuan Yang, Guo-Zhi Wu, Ying-Wen Li, Xiao Yu, Xiao-Li Ma, Yu-Ping Wang, Xu-Dong Hou, Qing-Hong Guo

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Xiao-Mei Ma, Yuan Yang, Guo-Zhi Wu, Ying-Wen Li, Xiao Yu, Xiao-Li Ma, Yu-Ping Wang, Qing-Hong Guo, Department of Gastroenterology, The First Hospital of Lanzhou University, Lanzhou 730000, Gansu Province, China

Xiao-Mei Ma, Yuan Yang, Guo-Zhi Wu, Ying-Wen Li, Xiao Yu, Xiao-Li Ma, The First Clinical Medical College, Lanzhou University, Lanzhou 730000, Gansu Province, China

Bao-Shun Yang, Xu-Dong Hou, General Surgery Ward 5, The First Hospital of Lanzhou University, Lanzhou 730000, Gansu Province, China

Corresponding author: Qing-Hong Guo, PhD, Chief Physician, Department of Gastroenterology, The First Hospital of Lanzhou University, No. 1 Donggang West Road, Chengguan District, Lanzhou 730000, Gansu Province, China. gqh@lzu.edu.cn

Abstract

BACKGROUND

Angiosarcoma is a highly malignant soft-tissue sarcoma derived from vascular endothelial cells that mainly occurs in the skin and subcutaneous tissues. Small-intestinal angiosarcomas are rare, and the prognosis is poor.

CASE SUMMARY

We reported a case of primary multifocal ileal angiosarcoma and analyze previously reported cases to improve our understanding of small intestinal angiosarcoma. Small intestinal angiosarcoma is more common in elderly and male patients. Gastrointestinal bleeding, anemia, abdominal pain, weakness, and weight loss were the common symptoms. CD31, CD34, factor VIII-related antigen, ETS-related gene, friend leukemia integration 1, and von Willebrand factor are valuable immunohistochemical markers for the diagnosis of small-intestinal angiosarcoma. Small-intestinal angiosarcoma most commonly occurs in the jejunum, followed by the ileum and duodenum. Radiation and toxicant exposure are risk factors for angiosarcoma. After a definite diagnosis, the mean and median survival time was 8 mo and 3 mo, respectively. Kaplan-Meier survival analysis showed that age, infiltration depth, chemotherapy, and the number of small intestinal segments invaded by tumor lesions were prognostic factors for small intestinal angiosarcoma. Multivariate Cox regression analysis showed that

chemotherapy and surgery significantly improved patient prognosis.

CONCLUSION
Angiosarcoma should be considered for unexplained melena and abdominal pain, especially in older men and patients with a history of radiation exposure. Prompt treatment, including surgery and adjuvant chemotherapy, is essential to prolonging patient survival.

Key Words: Angiosarcoma; Small intestine; Pathological features; Diagnosis; Prognosis; Case report

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Core Tip: Small intestinal angiosarcoma is a rare malignant soft tissue tumor. We report a primary multifocal ileal angiosarcoma with metastases to the adrenal gland and lumbar spine. The patient died 4 mo after surgical resection. Further, we collected relevant case reports and analyzed statistically. We concluded that small intestinal angiosarcoma tend to occur in elderly men. Melena and anemia were the most common symptoms. The diagnosis depended on microscopic morphology and immunohistochemistry. CD31, CD34, factor VIII-related antigen, ETS-related gene, friend leukemia integration 1, and von Willebrand factor were valuable diagnostic markers. Surgery and chemotherapy could improve the prognosis of patients.

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INTRODUCTION

Angiosarcoma is a rare malignant mesenchymal sarcoma that arises from vascular or lymphatic endothelial cells and accounts for only 1%-2% of all soft tissue sarcomas[1]. Angiosarcoma can invade any location in the body due to the widespread distribution of the blood and lymphatic systems[2]. Angiosarcoma has skin, visceral, and soft tissue subtypes, with visceral angiosarcoma accountings for 15%-47% and being more challenging to diagnose than the other subtypes[3]. Small intestinal angiosarcoma has a low incidence and presents with atypical abdominal pain, weight loss, nausea, vomiting, and gastrointestinal bleeding[4]. Various factors, including trauma, vinyl chloride, and radiation, have been implicated in the development of angiosarcoma. However, morbidity following exposure to these risk factors is rare. For example, a previous follow-up study showed that the overall risk of angiosarcoma after radiotherapy ranged from 0.01%-0.30%[5]. Timely diagnosis of small intestinal angiosarcoma is challenging owing to the diversity and non-specificity of the clinical symptoms, signs, and limited diagnostic methods, resulting in poor prognosis[6].

In this study, we report a case of primary small-intestinal angiosarcoma with lumbar and bilateral adrenal metastases. Furthermore, we retrospectively analyzed previously reported cases to explore the clinicopathological factors, diagnosis, treatment, and prognosis of small-intestinal angiosarcoma to further optimize the management and treatment of the disease.

CASE PRESENTATION

Chief complaints

A 70-year-old Chinese man presented with abdominal pain and melena for 4 mo.

History of present illness

The patient's symptoms had started four months earlier, accompanied by distension, constipation, poor appetite. There was no apparent cause. The patient had lost 15 kg.

History of past illness

The patient had a history of hypertension but no history of abdominal surgery, toxicity, or radiation exposure.

Personal and family history

The patient denied any family history of malignant tumors.

Physical examination

Physical examination revealed a chronically ill man. In addition, his abdomen was mildly swollen, with tenderness around the navel.

Laboratory examinations

Laboratory data revealed hemoglobin, hematocrit, and C-reactive protein levels of 10.1 g/dL, 30.8%, and 17.18 mg/L, respectively. The tumor marker levels were not elevated.

Imaging examinations

Electron gastroscopy and colonoscopy revealed no abnormalities. Computed tomography (CT) revealed that the part of the lower abdominal intestinal wall was significantly thickened with different degrees of enhancement in the arterial phase. In addition, bilateral adrenal masses and multiple soft-tissue nodules were noted in the right perirenal fascia (Figure 1). Magnetic resonance imaging (MRI) showed local abnormally enhanced nodules in the cauda equina at the L1/L2 Level; thus, metastasis was considered. Moreover, multiple nodules with abnormal signals in the bilateral adrenal area and right kidney were apparent, also leading to the consideration of metastasis (Figure 2). Electron enteroscopy revealed continuous periannulus ulcers 2.4-2.5 m above the ileocecal valve (Figure 3). The ulcer surface was covered with mucous moss, and the surrounding mucosa showed an irregular eminence, bled easily when touched, and had a hard texture.

PATHOLOGIC FINDINGS

A laparotomy was performed that revealed multiple grayish-red ulcerative tumors in the mucosa of the ileum, with a thin film of foul moss on the surface (Figure 4). An 8 cm × 6 cm ulcerative mass was also detected 2 m distal to the ligament of Treitz, resulting in intestinal obstruction. All lesions were resected and sent for pathological examination. Microscopically, the tumor tissues were hemorrhagic and necrotic. Spindle tumor cell infiltration was observed with round or spindle nuclei, thick chromatin, and mitotic images. Giant tumor cells were arranged in cords or scattered singly. Some tumor cells formed vascular channels with erythrocytes in the center, and parts of the lumen anastomosed with each other (Figure 5). Tumor cells infiltrated the subserosal layer. No tumor tissue was detected at the resection margins or perienteral lymph nodes. Immunohistochemistry results showed that the tumor cells were positive for CD31, vimentin, ETS-related gene (ERG) and p53, but negative for CK (Pan), epithelial membrane antigen (EMA), CD34, SMA, CD117, DOG1, S100, Melan A, HMB45, and MyoD1. The Ki-67 proliferation index was 40%.

FINAL DIAGNOSIS

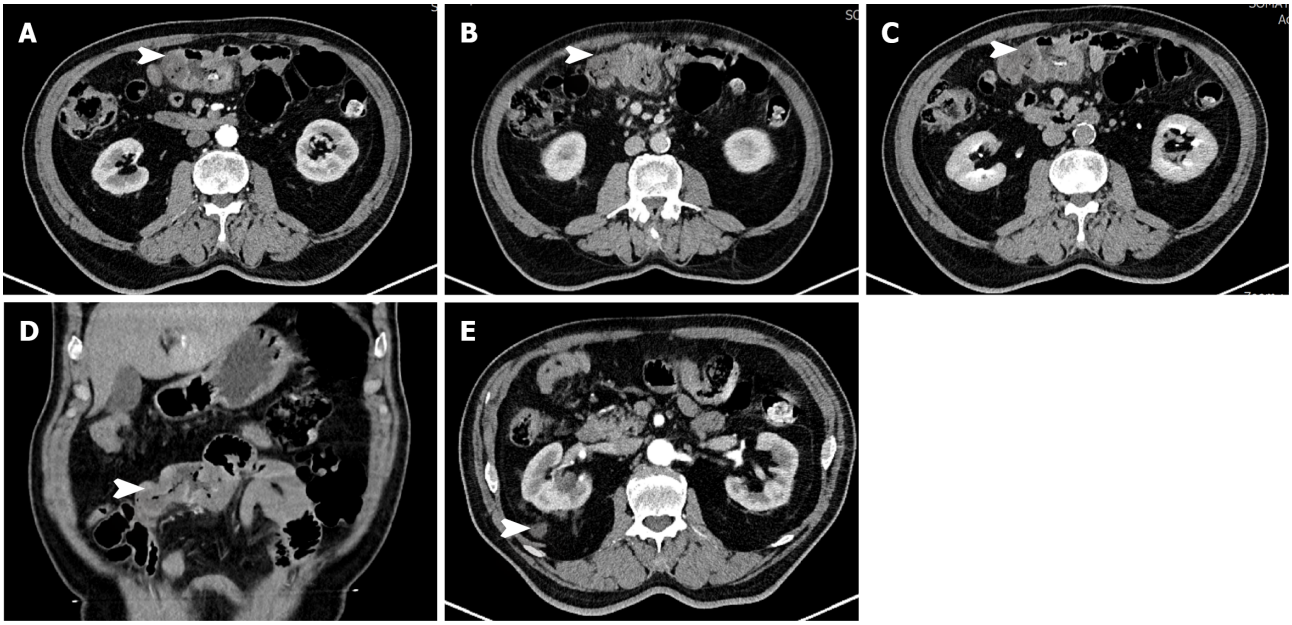
According to the pathological findings, the patient was definitely diagnosed with small intestinal angiosarcoma.

TREATMENT

The patient received R0 resection of small intestinal sarcoma with D2 Lymph node dissection and further functional end-to-end anastomosis. General supportive treatment was provided postoperatively. Further chemotherapy and molecular targeted therapy were suggested, but the patient declined owing to financial constraints.

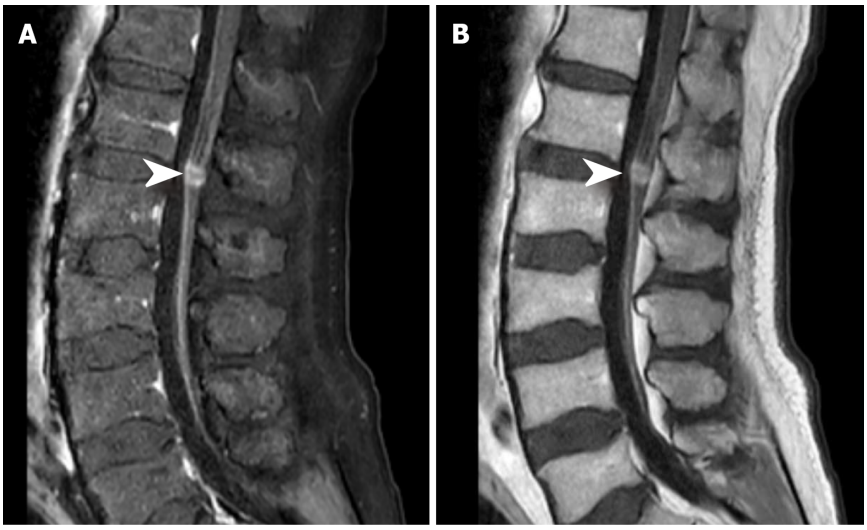
OUTCOME AND FOLLOW-UP

Following surgery, the patient's symptoms were relieved and there was no further melena. The patient was discharged following an improvement in his general condition. Shortly after discharge, the patient developed anorexia and diarrhea. However, the patient did not visit the hospital for review. He died four months later.



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Figure 1 Computed tomography showed segmental thickening of the small intestine (white arrow), with lesion enhancement in the arterial phase. A: Arterial phase; B: Venous phase; C: Balanced phase; D: Coronal plane; E: Adrenal masses.



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Figure 2 Magnetic resonance imaging showed local abnormally enhanced nodules (white arrow) at the L1/L2 Level in the cauda equina. A: T1 phase; B: T2 phase.

DISCUSSION

Literature review

We searched PubMed, Embase, Web of Science, and CNKI for cases of small intestinal angiosarcoma (updated until August 01, 2022). Only original reports published in English language were included. The search terms were ("small bowel" OR "small intestine" OR "small intestinal") AND ("adenocarcinoma" OR "hemangiosarcoma" OR "angiomatous sarcoma"). Including our case, a total of 82 cases was collected[1-4,6-77]. Of all the cases, 62 were primary, and 14 were secondary. The primary site of 6 cases could not be determined as the tumor lesions were found at multiple sites simultaneously. The basic clinical data of the patients are presented in Table 1. Prognostic information was available for 62 cases and an endpoint event (death) was observed in 52. SPSS software was used for statistical analyses. Survival curves were obtained using the Kaplan-Meier method (log-rank test). Univariate and multivariate analyses were performed using the Cox proportional hazards model. Categorical variables were compared using the chi-squared test. All tests were two-tailed, and statistical significance was set

Table 1 The basic clinicopathological factors of 82 collected cases

Characteristics	Number of patients	%
Gender		
Male	55	67.07
Female	27	32.93
Age		
≤ 65	34	41.46
> 65	47	57.32
NA	1	1.22
Race		
North America	35	42.68
European	17	20.73
Asia	26	31.71
other	4	4.88
Year		
≤ 2000	20	24.39
> 2010	62	75.61
Tumor origin		
Primary	62	75.61
Secondary	14	17.07
NA	6	7.32
Radiation history		
With	21	25.61
Without	61	74.39

NA: Not available.

at $P < 0.05$ unless otherwise stated. Data are presented as mean ± SD.

Age and gender characteristics of the patients

Of the 82 patients, 55 were men, and 27 were women, with a male-to-female ratio of 2.04:1.00. The ages of 55 men and 26 women were available (Table 2). The mean age of men was 64.44 years ± 14.92 years, with a range of 25-87 years; the mean age of women was 60.85 years ± 22.73 years, with a range of 20-92 years. The patients' age distribution is shown in Figure 6. There was no significant difference in age distribution between men and women ($P = 0.339$, Chi-square test).

Clinical symptoms and complications

The most frequent clinical symptoms (in order of frequency) were gastrointestinal bleeding (62.20%), anemia (57.32%), abdominal pain (37.80%), weakness (23.17%), weight loss (18.29%), shortness of breath (15.85%), nausea (13.41%), abdominal distention (12.20%), and loss of appetite (9.76%) (Table 3). Symptoms caused by angiosarcoma are challenging to distinguish from those of patients with gastrointestinal tumors, ulcers, and inflammatory diseases. The possibility of angiosarcoma should be considered in patients with unexplained gastrointestinal bleeding. The common abdominal complications were intestinal obstruction (18.29%), intestinal perforation (13.41%), intussusception (4.88%), and intraperitoneal hemorrhage (2.44%), which can result in an acute abdomen requiring emergency surgical management.

Detection of angiosarcoma lesions

The examination of the small intestine is difficult because of its anatomical location and structure. In recent years, the diagnostic rate of small-intestinal diseases has improved with the development of capsule endoscopy and enteroscopy. Among the cases collected, small bowel lesions or abnormalities were detected first by endoscopy in 23 cases, by CT in 12 cases, and capsule endoscopy in 6 cases. In

Table 2 Age distribution characteristics of patients

Gender	Number	Mean age	SD	Median age	Range of age
Female	26	60.85	22.73	70	20-92
Male	55	64.44	14.92	68	25-87
All	82	63.28	17.74	68	20-92

Table 3 Patients' symptoms during the disease

Symptoms	Number	Percentage (%)
Gastrointestinal bleeding	51	62.20
Anemia	47	57.32
Abdominal pain	31	37.80
Weak	19	23.17
Loss of weight	15	18.29
Short of breath	13	15.85
Nausea	11	13.41
Abdominal distention	10	12.20
Loss of appetite	8	9.76
Dizziness	5	6.10
Fever	3	3.66
Constipation	3	3.66
Chest pain	3	3.66
Back pain	3	3.66
Diarrhea	2	2.44
Syncope	2	2.44
Drowsiness	1	1.22
Peripheral edema	1	1.22
Lower limb weakness	1	1.22

addition, digestive tract radiography, MRI, barium meal, positron emission tomography (PET), and other examinations helped to detect lesions. In 26 cases, lesions were found by exploratory laparotomy, including those with an acute abdominal disease requiring emergency surgery and those in whim imaging and endoscopy examinations did not detect the lesion. In three cases, lesions were found on autopsy. The morphology of small intestinal angiosarcoma varies. Endoscopically, the tumors appear as deep or shallow ulcers, polyps, fungating lesion[22], nodules, huge masses, superficial elevations, depressions[48], or thickening and congestion of the small bowel wall[35]. Some lesions show varying degrees of hemorrhage[21,32,44,59] or are covered with filthy moss. On the CT scan, small intestinal angiosarcoma was characterized by segmental wall thickening of the small intestine[10,12,58,75,76], apple core lesion[11] and occupying lesion[6,20,33,67,70], with enlargement of the surrounding lymph nodes[52]. Necrosis was observed in the center of some lesions[33]. Contrast-enhanced CT scans showed different degrees of enhancement[4,57,61].

Diagnosis of small intestinal angiosarcoma

The diagnosis of angiosarcoma depends mainly on morphological characteristics and immunohistochemistry. Abnormal and malignant endothelial cells are the hallmarks of angiosarcoma and can be round, polygonal, spindle-shaped, or epithelioid in appearance. Well-differentiated angiosarcoma presents as well-formed vessels, papillary vascular spaces, or anastomotic narrow vascular channels with visible red blood cells in the lumen[29]. Poorly differentiated angiosarcomas are solid tumors characterized by continuous sheets of malignant cells[29]. Local necrosis and bleeding of tumor tissue are common. Table 4 presents the expression of immunohistochemical markers in the collected cases. CD31, CD34, factor VIII-related antigen (VIII), ERG, friend leukemia integration 1 (Fli-1), and von

Table 4 Characteristics of immunohistochemistry results

Pathological markers	Total	Positive	Negative
CD31	49	49	0
CD34	40	30	10
Vimentin	28	28	0
VIII	29	26	3
ERG	10	10	0
FLI-1	6	6	0
von Willebrand factor	2	2	0
EMA	17	1	16
SMA	10	0	10
CD117	10	0	10
Desmin	11	0	11
S100	28	1	27

VIII: Factor VIII-related antigen; ERG: ETS-related gene; FLI-1: Friend leukemia integration 1; EMA: Epithelial membrane antigen; SMA: Smooth muscle actin.

Willebrand factor (vWF) are important immune-positive markers of angiosarcoma. CD31 (49/49), ERG (10/10), FLI-1 (6/6), and vWF (2/2) were all positive in the stained cases. CD34 had a sensitivity of 75% (30/40), and VIII had a sensitivity of 89.7% (26/29). Besides, the immunohistochemistry results for EMA (16/17), SMA (10/10), CD117 (10/10), desmin (11/11), and S100 (27/28) were mostly negative in the collected angiosarcoma cases.

Distribution, characteristic, and metastasis of the lesions

The location of the small intestinal angiosarcoma, in descending order, was jejunum (28.0%), ileum (19.5%), duodenum (12.2%), whole small intestine (12.2%), duodenum/jejunum (11.0%), jejunum/ileum (6.1%), and unspecified small intestine (11.0%) (Table 5). Of the 76 cases that reported a definite location of the lesion, 42 cases invaded the jejunum (55.3%, A + D + E), 40 cases invaded ileum (52.6%, B + D + E + F) and 29 cases invaded duodenum (38.2%, C + D + F). There were 49 cases (64.5%) involving a single segment of the small intestine (A + B + C), 14 cases (18.4%) involving two segments of the small intestine (E + F), and 10 cases (13.2%) involving the entire small intestine. The characteristics of angiosarcoma lesions are shown in Table 6. Angiosarcomas of the small intestine tend to be multifocal (multifocal/single focal = 1.8). The size of the lesions varied, with a maximum diameter of < 40 mm for 38 Lesions and > 40 mm for 14 Lesions. The largest reported lesions can be up to 240 mm in diameter [33]. In two cases, the tumor lesions showed diffuse distribution[23,40]. Angiosarcoma lesions present as ulcer type (15.66%), superficial type (9.64%), diffuse infiltration type (7.23%), and protrusion (49.4%) types, including mass, polyp, mushroom type, and so on. Microscopically, the lesions invaded the mucosa in 3 cases, submucosa in 4 cases, muscularis propria in 5 cases, and serosa in 7 cases. In one case, the tumor lesion was located under the serosa. Among the 62 cases of primary small intestinal angiosarcoma, 35 cases (56.5%) had distant metastasis, 23 cases (37.1%) had no distant metastasis, and the other 4 (6.5%) were not specified (Table 7). The most frequent metastatic sites were the lung (22.6%), liver (21.0%), large intestine (21.0%), spleen (8.1%), bone (8.1%), pleural (6.5%) and stomach (6.5%). Of the 14 sary cases, 4 were primary in the skin of the head and face, 4 in the liver, 4 in the spleen, and one each in the pleuropulmonary, thyroid, sternocleidomastoid muscle, and rectum. Systemic examination and careful exploration are necessary for patients with angiosarcoma to prevent missing multiple or metastatic lesions.

Risk factors

A total of 21 cases had a clear history of radiation, including 15 women and 6 men (Table 8). There were 20 cases with a history of radiation therapy for tumors, and the remaining 1 had 30 years of severe occupational exposure to radiation and polyvinyl chloride. The time from radiation exposure to diagnosing small intestinal angiosarcoma fluctuated from 7 years to 45 years, with an average of 16.12 years ± 10.05 years. The radiation sites were located in the pelvis in 16 cases, chest in 2 cases, abdomen in 1 case, and neck in 1 case. The radiation dose ranged from 15 Gray to 60 Gray, with a mean of 48.03 Gray ± 14.18 Gray. Among the female patients, 10 were treated with radiation for uterine tumors, 2 for breast cancer, 2 for ovarian tumors, and 1 for colon cancer; of the male patients, 2 for prostate cancer, 1

Table 5 Location of small intestinal angiosarcoma

Tag	Location	Number	Percentage (%)
A	Jejunum	23	28.0
B	Ileum	16	19.5
C	Duodenum	10	12.2
D	Duodenum, Jejunum and Ileum	10	12.2
E	Duodenum and Jejunum	9	11.0
F	Jejunum and Ileum	5	6.1
G	Unspecified small intestine	9	11.0

Table 6 Characteristics of small intestinal angiosarcoma lesions

Characteristics	Number	%
Size		
≤ 40 mm	38	45.78
> 40 mm	14	16.87
Diffuse	2	2.41
NA	29	34.94
Small intestinal lesions		
Single focal	26	35.6
Multifocal	47	64.4
NA	9	
Pathological morphology		
Ulcerative	13	15.66
Protuberant	41	49.4
Superficial	8	9.64
Diffuse infiltrating	6	7.23
NA	15	18.07
Infiltration depth		
Mucosa	3	3.61
Submucosa	17	20.48
Muscularis propria	7	8.43
Serosa	17	20.48
Under the serosa	1	
NA	39	46.99

NA: Not available.

for abdominal lymphoma, 1 for tonsil cancer, and 1 for pelvic chondrosarcoma. In addition, a 45-year-old male patient had a history of hemodialysis for up to 21 years due to chronic renal insufficiency[46], and a 72-year-old male patient worked in the construction industry and may have had a long history of toxicological exposure[9].

Treatment

Treatment modalities were available for 74 cases among the reported cases. Among them, 42 patients (51.2%) underwent surgical resection only; 12 patients (14.6%) underwent surgical resection and chemotherapy; 11 patients (13.4%) received conservative treatment or no treatment; 5 patients (6.1%)

Table 7 Distant metastatic site of primary small intestinal angiosarcoma

Distant metastasis	Number	%
No metastasis	23	37.1
Lung	14	22.6
Liver	13	21.0
Large intestine	13	21.0
Spleen	5	8.1
Bone	5	8.1
Pleural	4	6.5
Stomach	4	6.5
Bladder	3	4.8
Kidney	2	3.2
Vein	2	3.2
Abdominal wall	2	3.2
Gallbladder	2	3.2
Pancreas	1	1.6
Heart	1	1.6
Adrenal gland	1	1.6
Pelvic cavity	1	1.6
Brain	1	1.6
Oropharynx	1	1.6
Diaphragm	1	1.6
NA	4	6.5
All	62	100%

NA: Not available.

received chemotherapy only; one underwent surgical resection, chemotherapy, and radiation therapy; one was treated with chemotherapy, radiotherapy, and immunotherapy; and one was treated with argon plasma coagulation. Of the patients who received chemotherapy, 2 with doxorubicin; 1 with paclitaxel plus carboplatin; 1 with adriamycin, vincristine, dacarbazine and Cytoxan; 1 with liposomal non-pegilated doxorubicin and Ifosfamide. Bevacizumab was the immunotherapy drug. Some patients required repeated blood transfusion treatment owing to anemia caused by chronic gastrointestinal blood loss[3,28].

Prognostic factors

We collected the survival time and status of 62 patients and performed a prognosis analysis. The mean survival of patients with small intestinal angiosarcoma was 234.77 d ± 41.88 d, with a range of 3 d to 3 years. Median survival time was 90.00 d ± 20.56 d. Respiratory failure, hemorrhagic shock, and multiple metastases were common causes of death. Kaplan-Meier survival analysis showed that age (P = 0.033), infiltrating depth (P = 0.038), chemotherapy (P = 0.025), and the number of small intestinal segments tumor involved (P = 0.020) were prognostic factors for small intestinal angiosarcoma (Figure 7). Sex, risk factors, acute abdomen, tumor origin, tumor size, number of tumor lesions, and distant metastasis had no significant effect on patient prognosis (P > 0.100). In the COX regression survival analysis, infiltration depth was eliminated owing to a large amount of missing data. Univariate COX regression analysis showed that age > 65 years (P = 0.047) and tumor lesions involving three whole segments (P = 0.020) of the small intestine, without chemotherapy (P = 0.032) were risk factors for small intestinal angiosarcoma (Table 9). We included factors with P < 0.100 in the univariate COX regression analysis into the multivariate analysis to avoid missing important influencing factors. The results showed that chemotherapy [P = 0.038, HR: 0.442 (0.205-0,956)], and surgery [P = 0.028, HR: 0.407 (0.182--0.908)] effectively improved patient prognosis (Table 10).

Table 8 Analysis of risk factors for small intestinal angiosarcoma

Gender/age (yr)	Time[1] (yr)	Cause of radiation	Radiation area	Dose (Gray)
F/66	20	Uterus cancer	Pelvis	NA
F/69	7	Adenocarcinoma of uterus	Pelvis	NA
F/50	14	Adenocarcinoma of uterine body	Pelvis	55.6
F/76	7	Adenocarcinoma of uterine body	Pelvis	45.1
F/78	10	Endometrial cancer	Pelvis	55.5
F/51	9	Adenocarcinoma of cervix	Pelvis	50
F/80	20	Squamous cell carcinoma of cervix	Pelvis	55
F/61	20	Squamous cell carcinoma of cervix	Pelvis	NA
F/NA	8	Cervical cancer	Pelvis	NA
F/72	24	Leiomyosarcoma of uterus	Pelvis	NA
F/26	14	Dysgerminoma of ovary	Pelvis	48
F/66	8	Ovarian cancer	Pelvis	60
F/88	18	Breast cancer	Chest	NA
F/37	NA	Breast cancer	Chest	NA
F/92	12	Colon cancer	Pelvis	NA
M/82	NA	Prostate cancer	Pelvis	NA
M/80	NA	Prostate cancer	Pelvis	NA
M/73	NA	Squamous cell carcinoma of tonsil	Neck	NA
M/63	45	Left lower abdominal lymphoma	Abdomen	15
M/57	8	Chondrosarcoma of right hemipelvis	Pelvis	NA
M/68	30	Occupational exposure	NA	Heavy

[1]Time: Duration from exposure to risk factors to diagnosis of small intestinal angiosarcoma.
M: Male; F: Female; NA: Not available.

DISCUSSION

Primary small intestinal malignancies are rare, accounting for < 2% of gastrointestinal tumors[36]. Small intestinal malignant tumors are often discovered late, due to their nonspecific symptoms and limited examination methods, resulting poor prognosis[51]. Gastrointestinal bleeding caused by small intestinal angiosarcoma is difficult to detect using routine gastroscopy and electronic colonoscopy[17]. CT, capsule endoscopy[25], PET[29], tagged red blood cell scanning[18], and push enteroscopy[28] may aid in the detection of small intestinal lesions. However, lesions were not detected in some patients after multiple examinations, thus necessitating surgical exploration. Even with endoscopic tissue biopsies, definitive diagnosis requires several attempts in some patients[9]. Thus, small intestinal angiosarcoma should be considered in patients with early abdominal symptoms, especially in older adults with melena, to avoid rapid disease development due to missed diagnosis.

Angiosarcoma is an aggressive tumor with high lymph node and peripheral metastases[51]. In our literature review, primary small intestinal angiosarcoma had a distant metastasis rate of at least 56.5%. Small intestinal angiosarcoma often metastasizes to the lungs, liver, large intestine, and spleens. Respiratory failure due to pulmonary metastases is a common cause of death in patients with small intestinal angiosarcoma (15 cases). There was one case of metastasis to an uncommon site, the right atrial appendage and right ventricular septum, with a survival of only 12 d[34]. Therefore, for patients diagnosed with angiosarcoma of the small intestine, systemic examinations, such as PET-CT, are recommended, with attention paid specifically to pulmonary metastases.

Depending on the degree of differentiation, angiosarcoma can develop and range from being a well-differentiated vascular form to a poorly differentiated solid tissue. The solid growth pattern of angiosarcoma consists of two cell types: Sheets of spindle-shaped or large, polygonal epithelioid-type cells with a high mitotic rate[29]. The specific angiosarcoma subtype consisting of epithelioid tumor cells is called epithelioid angiosarcoma[32]. Epithelioid morphology is typical, but it can also express endothelial-related markers, such as cytokeratin, leading to confusion with other entities, such as

Table 9 Results of univariate COX regression analysis

Factors	HR (98%CI)	*P* value
Gender (male/female)	1.395 (0.772-2.521)	0.270
Age (> 65/≤ 65)	1.803 (1.007-3.227)	0.047
Tumor origin (secondary/primary)	1.708 (0.789-3.696)	0.174
Tumor size(> 40 mm/≤ 40 mm)	1.265 (0.578-2.767)	0.557
Tumor lesion (multifocal/single focal)	1.365 (0.722-2.579)	0.338
Distant metastases (yes/no)	1.140 (0.573-2.269)	0.708
Acute abdominal disease (yes/no)	0.780 (0.424-1.435)	0.424
Surgery (with/without)	0.570 (0.296-1.100)	0.094
Chemotherapy (with/without)	0.473 (0.238-0.940)	0.032
Gastrointestinal bleeding (yes/no)	1.076 (0.604-1.915)	0.803
Tumor distribution		
Two segments/one segment	2.116 (0.975-4.593)	0.058
Whole intestine/one segment	2.473 (1.156-5.289)	0.020

Table 10 Results of multivariate COX regression analysis

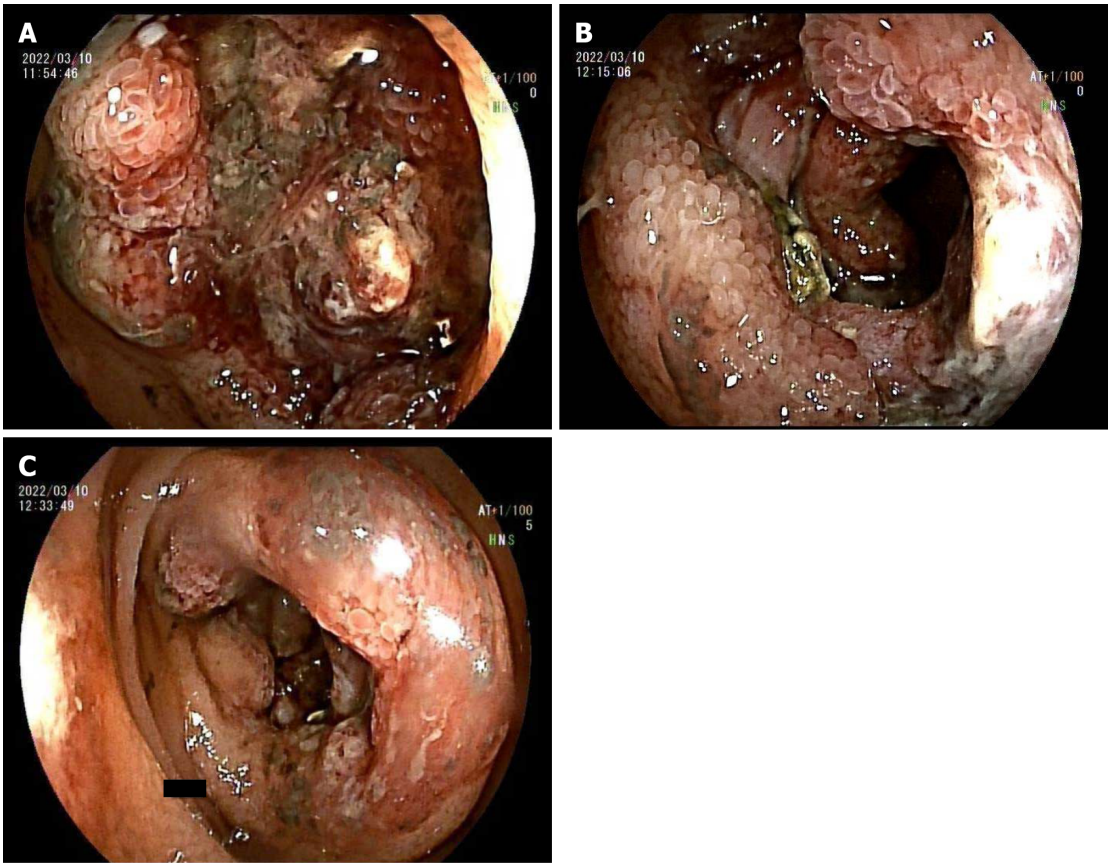
Factors	HR (98%CI)	*P* value
Chemotherapy (with/without)	0.442 (0.205-0.956)	0.038
Surgery (with/without)	0.407 (0.182-0.908)	0.028
Age (> 65/≤ 65)	1.944 (0.969-3.902)	0.061
Tumor distribution		
Two segments/one segment	0.434 (0.194-0.969)	0.042
Whole intestine/one segment	0.820 (0.323-2.086)	0.677

malignant melanoma, fibrosarcoma, mesothelioma, or sarcoma with epithelioid features (particularly gastrointestinal stromal tumors)[10,28].

Immunohistochemistry is essential for the diagnosis of angiosarcoma. Positive expression of endothelial markers, including CD31, CD34, factor VIII, ERG, Fli-1, and vWF, help define the vascular nature of the tumor[3]. CD31 and ERG show the highest positive detection rates. The specificity of CD34 is relatively low and is positively expressed in 60%-70% of gastrointestinal stromal tumors[78]. Vimentin is a marker of epithelial-mesenchymal transition, and its overexpression in tumors is closely related to accelerated growth, invasion, and poor prognosis[52]. Vimentin is also widely expressed in other tumors, including melanoma, malignant mesothelioma, and epithelioid sarcoma, thus lacking reliability in the differential diagnosis of angiosarcoma[3]. As a negative marker in angiosarcoma, S-100 proteins help differentiate angiosarcoma from carcinoma and melanoma[10]. EMA cannot be used definitively in the differential diagnosis of angiosarcoma as it can be positive for epithelioid angiosarcoma[3,52]. CD117 is commonly used to diagnose gastrointestinal stromal tumors[78]. However, previous studies have shown that > 50% of angiosarcomas are positive for CD117[79]. Additionally, epithelioid and some non-epithelioid angiosarcoma cases may express keratin[3,76].

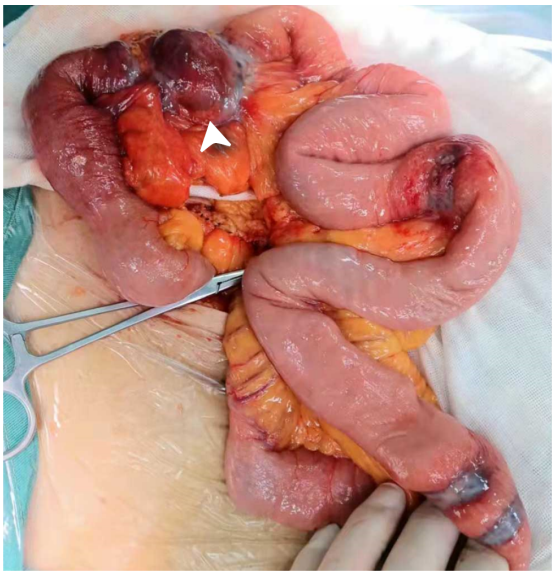
The prognosis for small intestinal angiosarcoma is poor, and the one-year survival rate was only 20.8% among the cases reviewed in the present study. Old age, infiltration depth, and involvement of two or all segments of the small intestine are risk factors for poor prognosis. Multivariate Cox regression analysis showed that surgery and chemotherapy can significantly improve the prognosis of patients with small intestinal angiosarcoma. In addition to surgical resection and chemotherapy, nutritional support, medication or endoscopic hemostasis, blood transfusion, and other treatments are also important. Local radiotherapy is also an alternative treatment.

With the development and clinical application of molecular targeted drugs, molecular targeted therapy for tumors has become a research hotspot in medical oncology. Studies have shown that vascular endothelial growth factor (VEGF) and its receptor (VEGFR) are highly expressed in angiosarcoma. VEGF and VEGFR inhibitors or multi-tyrosine kinase inhibitors, including bevacizumab and pazopanib, are potential drug targets for angiosarcoma. Malignant vascular tumors, including



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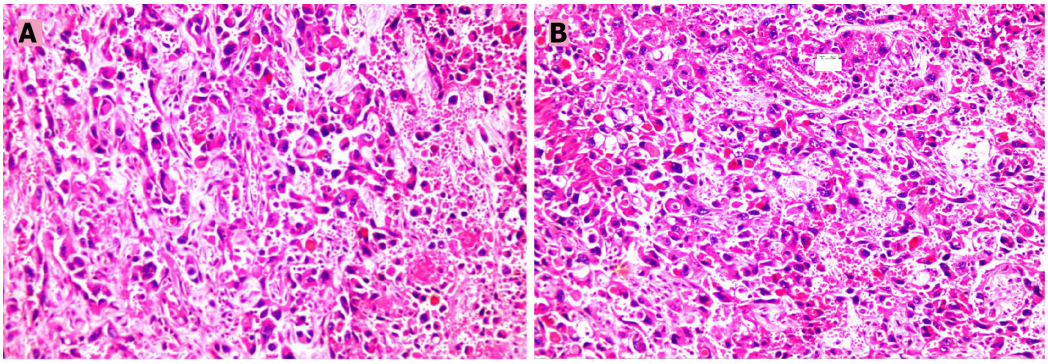
Figure 3 Electronic double-balloon enteroscopy. A-C: Electronic double-balloon enteroscopy showed continuous periannulus ulcers 2.4-2.5 m above the ileocecal valve, covered with mucous moss.



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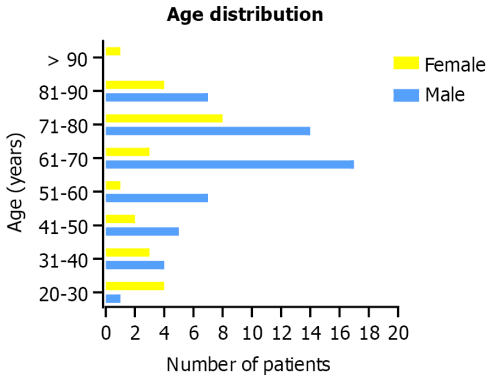
Figure 4 During the operation, multiple grey-red ulcerative tumors were observed in the ileum mucosa covered with moss. In addition, an 8 cm × 6 cm ulcerative mass (white arrow) resulted in intestinal obstruction.

angiosarcoma, express high levels of adrenergic receptors. Targeting these receptors with drugs such as protamine inhibited tumor growth in mouse vascular cell lines[80]. In addition, a few cases with cutaneous angiosarcoma showed significant responses to checkpoint inhibitors, including pembrolizumab, anti-PD-L1 antibody, and anti-CTLA-4 antibody[81]. However, existing immunotherapy



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Figure 5 Pathologic findings. A and B: Microscopically, spindle cell infiltration was observed with round or spindle-shaped nuclei. In some areas, tumor cells formed vascular channels with red blood cells in the middle (× 100).



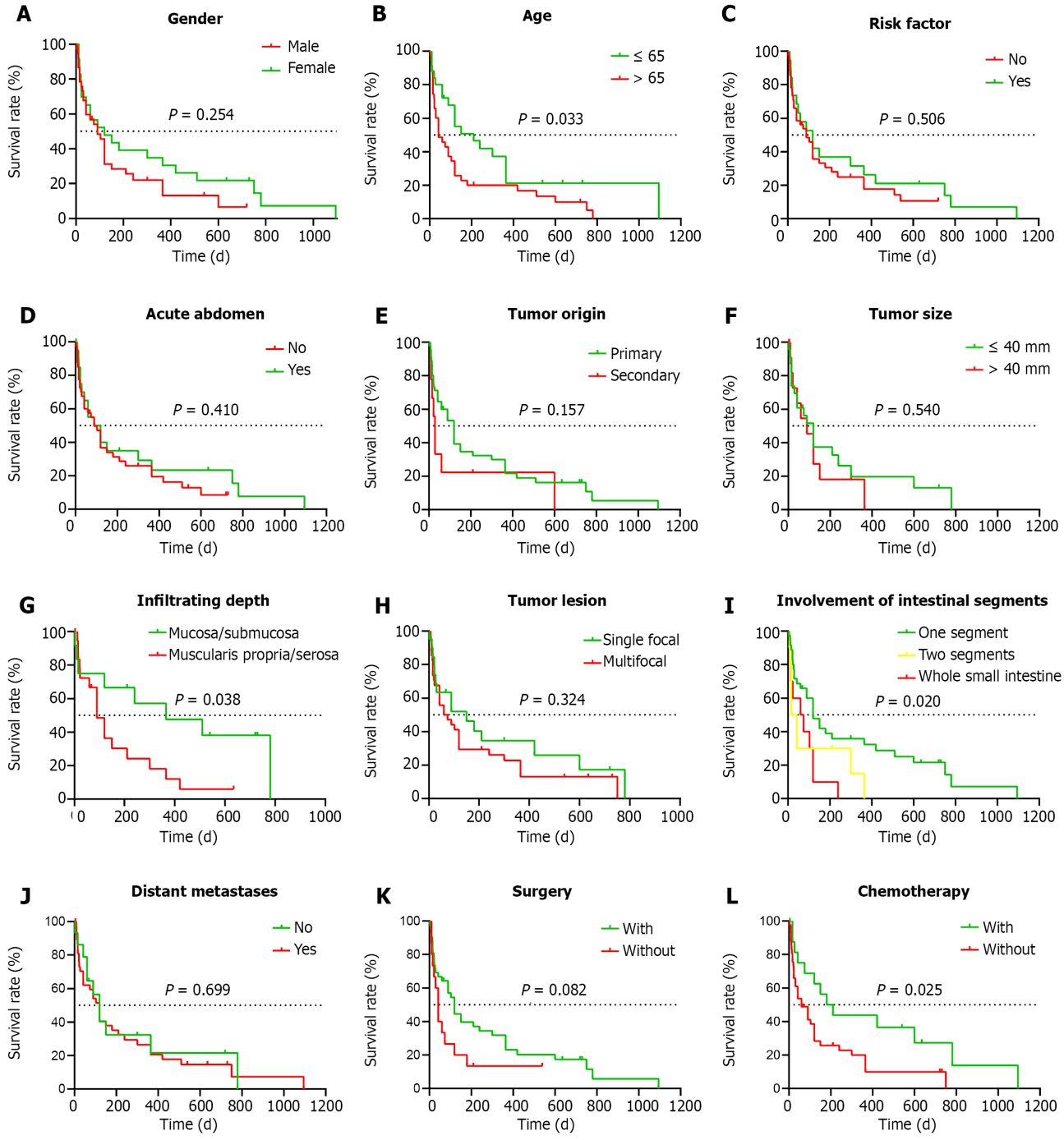
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Figure 6 Age distribution of male and female patients.

clinical trials mostly focused on cutaneous angiosarcoma, and relevant research on small intestinal angiosarcoma is lacking. Bevacizumab was administered to only one patient with angiosarcoma of the small intestine. However, due to the rapid progression of the patient's disease and failure to take drugs regularly, it was impossible to objectively evaluate its effect[17].

CONCLUSION

This study reported a case of multiple small intestinal angiosarcomas that resulted in intestinal obstruction with lumbar and bilateral adrenal metastases. Furthermore, we summarized the clinical features, diagnosis, treatment, and prognosis of 82 reported cases of small intestinal angiosarcoma. We found that small intestinal angiosarcoma occurred mainly in older men, and the most common symptom was gastrointestinal bleeding, which mainly manifested as melena. The main treatment methods were surgical resection and chemotherapy, which effectively improved patients' survival. This will help clinicians to understand small intestinal angiosarcomas and guide their clinical diagnosis and treatment. However, statistical bias is inevitable because of the small sample size. In addition, few clinical trials are related to chemotherapy and immunotherapy, and treatment methods are limited. Therefore, we expect that statistical analysis of larger samples and drug clinical trials will improve patients' clinical management and prognosis with small intestinal angiosarcoma.



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Figure 7 Kaplan-Meier survival analysis. A: Sex; B: Age; C: Risk factor; D: Acute abdomen; E: Tumor origin; F: Tumor size; G: Infiltrating depth; H: Number of tumor lesions; I: Intestinal segments involvement; J: Distant metastases; K: Surgery; L: Chemotherapy.

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FOOTNOTES

Author contributions: Ma XM contributed to data analysis and manuscript writing; Yang BS contributed to conceptualization and methodology; Yang Y contributed to data curation and visualization; Wu GZ contributed to data curation, writing-original draft preparation; Li YW, Yu X, and Ma XL contributed to literature search and screening; Wang YP and Hou XD contributed to revised the manuscript and approved the final version; Guo QH

contributed to supervision and final confirmation.

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Country/Territory of origin: China

ORCID number: Xiao-Mei Ma 0000-0003-1117-8309; Bao-Shun Yang 0000-0002-2772-694X; Yuan Yang 0000-0003-2212-4023; Guo-Zhi Wu 0000-0002-1223-1653; Yu-Ping Wang 0000-0003-0087-4771; Qing-Hong Guo 0000-0003-2456-7362.

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LETTER TO THE EDITOR

Discussion on gemcitabine combined with targeted drugs in the treatment of pancreatic cancer

Jun-Hao Huang, Wei Guo, Zhe Liu

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Jun-Hao Huang, Wei Guo, Zhe Liu, Department of Pancreatic-Biliary Surgery, The First Hospital of China Medical University, Shenyang 110001, Liaoning Province, China

Corresponding author: Zhe Liu, MD, Doctor, Professor, Surgeon, Department of Pancreatic-Biliary Surgery, The First Hospital of China Medical University, No. 155 Nanjing North Street, Heping District, Shenyang 110001, Liaoning Province, China. liuzhe4321@126.com

Abstract

Pancreatic cancer is a malignant tumor with poor prognosis. The treatment of pancreatic cancer depends on the tumor stage and type, and includes local treatment (surgery, radiotherapy and ablation intervention) and systemic therapy (chemotherapy, targeted therapy and immunotherapy). We read with great interest the review "Effective combinations of anti-cancer and targeted drugs for pancreatic cancer treatment" published on *World J Gastroenterol* and intended to share some of our perspectives in pancreatic cancer treatment. This review presents the therapeutic effects of the combination of gemcitabine and targeted drugs, which gives us a deeper insight into the combination treatments for pancreatic cancer.

Key Words: Pancreatic cancer; Chemotherapy; Targeted therapy; Gemcitabine; Drug; Combination

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Core Tip: In terms of the choice of chemotherapy regimen for pancreatic cancer, multi-drug chemotherapy is often applied in clinical practice. In general, the combination of chemotherapy and targeted therapy have better efficacy, but whether the combination of the two schemes is more effective than chemotherapy alone requires further investigations.

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TO THE EDITOR

We have read with great interest the review "Effective combinations of anti-cancer and targeted drugs for pancreatic cancer treatment" published on *World J Gastroenterol*[1]. As is known, gemcitabine alone had limited efficacy in the treatment of pancreatic cancer. This review reported that gemcitabine in combination with targeted agents, like the combination of gemcitabine and Chk1 inhibitor, gemcitabine and KRAS antibody/MEK inhibitor, gemcitabine and autophagy inhibitor, had better efficacy. In addition, the combination of targeted drugs also resulted in better clinical outcome, such as ERK and autophagy inhibitors; ERK, Chk1, and autophagy inhibitors; 2-deoxyglucose and MEK inhibitors; replication stress response and autophagy inhibitors; and immune checkpoint and autophagy inhibitors. It is interesting to note that some natural products, such as cucurbitacin B and glaucarubinone, also had better therapeutic effects in pancreatic cancer when combined with other drugs or with other natural products[1]. We agree with the authors that the combination could improve the therapeutic efficacy in patients with pancreatic cancer. Based on this review and our clinical experience we here share some of perspectives about pancreatic cancer treatment.

According to the National Comprehensive Cancer Network guidelines, there are many methods of chemotherapy used for treating pancreatic cancer, including multi-drug chemotherapy. The current standard first-line treatment regimen for metastatic pancreatic cancer includes gemcitabine and albumin-bound paclitaxel or modified FOLFIRINOX (leucovorin, fluorouracil, irinotecan, oxaliplatin)[2, 3]. A study of 861 untreated patients with metastatic pancreatic cancer has reported a better efficacy of gemcitabine and albumin-bound paclitaxel compared with gemcitabine [median survival, 8.5 *vs* 6.7 mo; hazard ratio = 0.72, 95% confidence interval (CI): 0.620-0.83; $P < 0.001$)[4]. In the current clinical practice, gemcitabine is rarely used alone. This review pointed out that gemcitabine was used for chemotherapy combined with various targeted drugs, but did not mention whether gemcitabine and albumin-bound paclitaxel combined with targeted drugs have better effects, which is important in the treatment of this cancer and needs to be identified. Targeted drugs combined with gemcitabine may have variable efficacy for different stages of pancreatic cancer. The review reported that gemcitabine combined with some targeted drugs yielded better clinical outcome, however, in our opinion, the combination is not always as effective as we expect, which may be worth discussion. The phase III LAP07 trial in 2016 investigated the clinical value of erlotinib combined with gemcitabine in patients with locally advanced pancreatic cancer. The median overall survival of the patients treated with gemcitabine alone was 13.6 mo (95%CI: 12.3-15.3 mo), while the patients receiving gemcitabine combined with erlotinib had a median overall survival of 11.9 mo (95%CI: 10.4-13.5 mo). The combination *vs* gemcitabine alone, despite good adherence, failed to improve survival and was associated with increased grade 3 hematologic, digestive, and skin toxicity[5]. CONKO-006 was a randomized double-blinded phase IIb study designed to evaluate the efficacy of the combination of gemcitabine and sorafenib compared with gemcitabine and placebo in patients with pancreatic adenocarcinoma with postsurgical R1 residual status. The results indicated that there were no differences in recurrence-free survival nor overall survival between the two groups[6]. The exact mechanism by which the combination of drugs could be less effective than gemcitabine alone is difficult to explain and may be related to the greater toxicity of combination drugs. An open-label, multicenter, randomized phase II trial evaluated gemcitabine plus afatinib *vs* gemcitabine alone for metastatic pancreatic cancer. Median overall survival was 7.3 mo with gemcitabine plus afatinib *vs* 7.4 mo with gemcitabine alone. Adverse events like diarrhea and rash were more frequent with gemcitabine plus afatinib[7]. In brief, these studies remind us that different combinations of chemotherapeutic drugs and targeted drugs may have different effects for various stages of pancreatic cancer. In conclusion, this review has led us to focus on new options of pancreatic cancer treatment, which is significant in guiding the clinical pancreatic cancer treatment and pointing out the direction for future research.

FOOTNOTES

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ORCID number: Jun-Hao Huang 0000-0001-6501-599X; Wei Guo 0000-0003-0375-0950; Zhe Liu 0000-0003-2950-3966.

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REVIEW

Cytotoxic synergism of *Clostridioides difficile* toxin B with proinflammatory cytokines in subjects with inflammatory bowel diseases

Gabrio Bassotti, Alessandro Fruganti, Fabrizio Stracci, Pierfrancesco Marconi, Katia Fettucciari

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Gabrio Bassotti, Department of Medicine and Surgery, Gastroenterology, Hepatology & Digestive Endoscopy Section University of Perugia Medical School, Piazza Lucio Severi, Perugia 06132, Italy, and Santa Maria della Misericordia Hospital, Gastroenterology & Hepatology Unit Perugia 06156, Italy

Alessandro Fruganti, School of Biosciences and Veterinary Medicine, University of Camerino, Matelica 62024, Italy

Fabrizio Stracci, Medicine and Surgery, Hygiene and Public Health Section, University of Perugia, Perugia 06123, Italy

Pierfrancesco Marconi, Katia Fettucciari, Medicine and Surgery, Biosciences & Medical Embryology Section, University of Perugia, Perugia 06132, Italy

Corresponding author: Gabrio Bassotti, MD, PhD, Associate Professor, Department of Medicine and Surgery, Gastroenterology, Hepatology & Digestive Endoscopy Section University of Perugia Medical School, Piazza Lucio Severi, Perugia 06132, Italy, and Santa Maria della Misericordia Hospital, Gastroenterology & Hepatology Unit Perugia 06156, Italy. gabassot@tin.it

Abstract

Clostridioides difficile (*C. difficile*) is progressively colonizing humans and animals living with humans. During this process, hypervirulent strains and mutated toxin A and B of *C. difficile* (TcdA and TcdB) are originating and developing. While in healthy subjects colonization by *C. difficile* becomes a risk after the use of antibiotics that alter the microbiome, other categories of people are more susceptible to infection and at risk of relapse, such as those with inflammatory bowel disease (IBD). Recent *in vitro* studies suggest that this increased susceptibility could be due to the strong cytotoxic synergism between TcdB and proinflammatory cytokines the tumor necrosis factor-alpha and interferon-gamma (CKs). Therefore, in subjects with IBD the presence of an inflammatory state in the colon could be the driver that increases the susceptibility to *C. difficile* infection and its progression and relapses. TcdB is internalized in the cell *via* three receptors: chondroitin sulphate proteoglycan 4; poliovirus receptor-like 3; and Wnt receptor frizzled family. Chondroitin sulphate proteoglycan 4 and Wnt receptor frizzled family are involved in cell death by apoptosis or necrosis

depending on the concentration of TcdB and cell types, while poliovirus receptor-like 3 induces only necrosis. It is possible that cytokines could also induce a greater expression of receptors for TcdB that are more involved in necrosis than in apoptosis. Therefore, in subjects with IBD there are the conditions: (1) For greater susceptibility to *C. difficile* infection, such as the inflammatory state, and abnormalities of the microbiome and of the immune system; (2) for the enhancement of the cytotoxic activity of TcdB +Cks; and (3) for a greater expression of TcdB receptors stimulated by cytokines that induce cell death by necrosis rather than apoptosis. The only therapeutic approach currently possible in IBD patients is monitoring of *C. difficile* colonization for interventions aimed at reducing tumor necrosis factor-alpha and interferon-gamma levels when the infection begins. The future perspective is to generate bacteriophages against *C. difficile* for targeted therapy.

Key Words: Inflammatory bowel diseases; *Clostridioides difficile* infection; Cytokines; Tumor necrosis factor-alpha; Interferon-gamma; Necrosis; Apoptosis; Cytotoxic synergism

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Core Tip: *Clostridioides difficile* is an opportunistic pathogen that is progressively increasing worldwide. Patients with inflammatory bowel diseases are particularly susceptible due to altered immunological status and the therapies adopted that favor intestinal dysbiosis and colonization by *Clostridioides difficile*. Recent *in vitro* studies also suggest that the infection might be favored by the strong cytotoxic synergism between *Clostridioides difficile* toxin B and proinflammatory cytokines, thetumor necrosis factor-alpha and interferon-gamma. The therapeutic approaches are still limited, and those presently available rely on antibiotic therapy and fecal transplantation.

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INTRODUCTION

Clostridioides difficile (*C. difficile*)[1] is a gram-positive, anaerobic, and spore-forming bacterium[2-6], responsible for the most widespread healthcare-associated infection worldwide[7-11]. In the United States every year about 500000 individuals become infected, leading to approximately 29000 deaths, and in Europe there are 124000 cases of infected individuals per year with a mortality rate ranging from 3% to 30%[7-11]. *C. difficile* infection (CDI) accounts for more than 15%-25% of all opportunistic gastrointestinal infections[7-11]. The rate of *C. difficile* colonization is about 18%-90% of healthy infants in relation to infant age[12], and 4%-15% of healthy individuals[13]. Its transmission occurs by the fecal-oral route[6,14], mainly by spores. Hospitals and community healthcare settings are an important source of infection due to the presence of *C. difficile*-infected patients[15,16]. The latter create a microenvironment highly contaminated by *C. difficile* spores extremely resistant to common strong disinfectants and radiation[17-19].

The clinical manifestations of CDI vary from asymptomatic carriage or mild self-limiting diarrhea to pseudomembranous colitis, with complications including toxic megacolon, fulminant infection and death[2-5,10]. The disease strictly depends on *C. difficile* germination and the release of three toxins[2-5,10]. The Rho-glycosylating *C. difficile* toxins A (TcdA) and B (TcdB) are major toxins that are clearly responsible for diarrhea and colitis[2-5,10]. In addition, 5%-30% of clinical *C. difficile* strains produce a binary autoprotease domain-ribosylating toxin, called the *C. difficile* binary toxin (CDT), that modifies actin[20,21].

It has previously been highlighted how the continuous progressive spread of *C. difficile* in the anthropized environment and the ability to develop more virulent strains will allow it to colonize most of the human population in the near future[22,23]. Among the subjects who will be more progressively colonized/infected with the progressively expanding *C. difficile* are those with inflammatory bowel diseases (IBD)[24-28], a category which is increasing in number in both Western and Eastern countries [29,30,31].

The progressive *C. difficile* endemic spread and the growing number of IBD subjects[25,29,32,33] are already interacting, as evidenced by studies showing how the rate of CDI cases in patients with IBD has increased by approximately four times in recent years[25]. It is therefore important to understand the

molecular and pathogenic events by which *C. difficile* colonizes and infects and how these can impact subjects with IBD characterized by a profound alteration of the microbiome, of the immune system, and of the inflammatory response.

C. DIFFICILE

CDI and clinical manifestations

CDI causes nosocomial/antibiotic-associated and community healthcare diarrhea with abdominal pain and cramps[6,10,32,33]. Colitis without pseudomembrane formation features watery diarrhea, trace blood in stool, nausea, abdominal pain, malaise, anorexia, low-grade fever, dehydration, pyrexia, and leucocytosis[6,10,32,33]. Clinical manifestations of pseudomembranous colitis consist of abdominal cramps, watery diarrhea with dehydration, hypoalbuminemia, and increased serum proteins, mucus, and inflammatory cells. Sometimes plaques (pseudomembranes) are found in the colorectal mucosa[6,10,32,33]. Fulminant colitis, observed in about 3% of CDI patients, induces serious complications such as perforation, prolonged ileus, megacolon, and death[6,10,32,33]. CDI may not be limited to the colon, and extra-colonic manifestations have been reported, including small bowel disease with the formation of pseudomembranes on the ileal mucosa, bacteremia, reactive arthritis, appendicitis, intra-abdominal abscesses, osteomyelitis, and empyema[34,35] In recent years, a significant rise in cases of fulminant colitis, which results in the development of symptoms, multiple organ failure, and increased mortality, has been associated with hypervirulent strains of *C. difficile*[6,10,32,33]. The disease strictly depends on *C. difficile* production and release of three toxins[2-5,10]. TcdA and TcdB are primarily responsible for clinical manifestations of disease[2-5,10]. However, 5%-30% of *C. difficile* clinical strains produce CDT, which contributes to disease by means of actin modification[20,21].

TcdA and TcdB: molecular structure and their receptors

TcdA and TcdB are single chain proteins, with a molecular weight of 308 kDa for TcdA and 270 kDa for TcdB[2-5,32]. Tcds show 48% sequence identity and 66% sequence similarity, where the diversity of sequence is mainly limited to the C-terminal binding domain. TcdA and TcdB are constituted by four domains, each characterized by specific biological and functional properties: (1) A glucosyltransferase N-terminal domain (GTD); (2) An autoprotease domain; (3) A pore forming and translocation domain; and (4) A C-terminal binding repetitive oligopeptides domain (CROP)[2-5,32]. The CROP domain and other amino acids outside this domain allow the binding of Tcds to the cells for subsequent internalization[2-5]. Although TcdA and TcdB CROPs display the solenoid fold, they present distinct spatial and sequential arrangements of their repeat units. This agrees with findings that suggest that both TcdA and TcdB bind to different receptors[2-5,32]; therefore, TcdA and TcdB do not follow the rule of one toxin-one receptor[2-532,36].

Two different cell surface receptors have been proposed for TcdA: rabbit sucrase isomaltase and gp96 [2-5,32,36]. Since many cells and tissues that are sensitive to TcdA do not express sucrase isomaltase and cells lacking gp96 are only partially resistant to TcdA intoxication, TcdA could bind to other receptor structures[2-5,32,36]. Three different receptors have been identified for TcdB[2-5,32,36]: Chondroitin sulphate proteoglycan 4 (CSPG4); poliovirus receptor-like 3 (PVRL3); and Wnt receptor frizzled family (FZD; FZD1, 2 and 7)[2-5,32,36]. TcdB binding to the CSPG4 receptor in HeLa and HT29 cells induces cell rounding and apoptosis at picomolar concentrations of TcdB but induces necrosis at higher concentrations of TcdB[32,36-38]. TcdB binding to the PVRL3 receptor induces necrosis at high doses (the nanomolar range) of TcdB[32,36-39]. TcdB binding to the FZD receptors induces cytopathic effects and apoptosis at picomolar concentrations of TcdB, indicating that FZD functions as an alternative receptor to CSPG4[36-40]. A further significant distinctiveness of TcdB is that it can bind to the membrane receptor with amino acid sequences that extend beyond the CROP sequences[36-40].

This description, in agreement with recent studies, indicates that TcdA and TcdB use more than one receptor for cell binding and uptake and highlights how the heterogeneity of the TcdB-bound receptors may impact on the diversity of the effects in relation to the receptor binding, concentrations of TcdB, and cell types[36-43]. It is possible that TcdB may utilize multiple receptors to broaden the selection of mammalian cells it can target[36-41]. Further, TcdB variants are highly diverse for their receptor preference, with relevant implications on colonic pathology[20,32,36,41].

The role played by CROP domains is also demonstrated by the fact that antibodies directed toward the CROP domains of TcdA and TcdB prevent uptake[2-5,36], and excess of the TcdA CROP domain compete with TcdA for cell binding[2-5,36]. However, TcdA and TcdB that lack CROP domains are still capable of internalization by the cells[2-5,36]. To understand some important aspects of the pathogenic strategy of *C. difficile* it is necessary to know what Tcd receptors are expressed on different cell types. Receptors for Tcds do not have well-defined molecular structures[20,32,36-40]; they are likely formed by a complex configuration of the polysaccharide chains that are recognized by the TcdA or TcdB binding domains with a lectin-like structure[42]. Furthermore, TcdA and TcdB have some important features of intrinsically disordered proteins[44] that allow Tcds to modify their conformation to adapt and more efficiently bind to the target structure of Tcd receptors[32,36-40]. The complex and intrinsically

disordered structural features of the Tcds allow them to bind very different cell types[2-5,32], such as the cells of surface epithelium of the human colon[43], colonocytes[45], hepatic cells[46], nervous cells [47], enteric glial cells (EGCs)[48], and cardiac cells[49].

It has been hypothesized that when *C. difficile* spores germinate into their vegetative forms and replicate that their susceptibility to the cytotoxic activity of macrophages, polymorphonucleates, and lymphocytes will increase[32,50-52]. These immune cells induce and increase the inflammatory response [3,50-53] characterized by secretion of several proinflammatory cytokines such as interleukin (IL)-1, IL-6, IL-8, interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α)[54-58]. *C. difficile* struggles against these immune cells by means of Tcds, capable of binding to receptors with different forms of carbohydrates that give rise to distinct structures. This result is obtained by the ability of TcdB to recognize three types of receptors[36-40] and by its intrinsically disordered structure[44]. Therefore, due to accidental molecular homology, Tcds could also be cytotoxic to other cell types not present in the infection site that express one or more of the Tcd receptors (*e.g.*, endothelial, hepatic, nervous, EGCs, and cardiac cells)[32,46-49]. Furthermore, the binding capability of Tcds toward colonocytes deepens the tissue damage within and beyond the submucosa. The subsequent damage to the muscle and enteric nervous system cells creates conditions to expel *via* diarrhea the *C. difficile* vegetative forms that rapidly become spores and to start a new infection cycle. It is also possible that if the Tcd receptor domain mutates, the pathogenicity of *C. difficile* may become more severe.

Tcd uptake and internalization

Tcds, after binding to their cell membrane receptors, promote their uptake by endocytosis[2-5,32]. Tcds differ in uptake pathways. TcdB uptake is mediated by clathrin[2], while TcdA uptake is mediated by PACSIN2/syndapin-II[2]. In the endocytic vacuole the progressive pH decrease promotes a Tcd conformational change that leads to translocation across the endosomal membrane of the catalytic domain for cleavage through vacuole pore formation[2-5,32]. Then, Tcds undergo autoprocessing by the cysteine protease domain that follows the N-terminal GTD[2-5,32] in a host-factor dependent manner (*e.g.*, inositolphosphates, mainly inositol hexakisphosphate)[2-5,32], releasing the GTD into the host cell cytosol[2-5,32].

Tcd intracellular effects

The Tcds GTD, by the monoglucosylation of the catalytic site of Rho-GTPases, inhibits their activity[2-5, 32]. The monoglucosylation of Rho-GTPases induces different effects *in vitro* and *in vivo*[2-5,32]. The effects mainly documented *in vitro* are: (1) Actin condensation, rearrangement of the cytoskeleton, and disruption of focal adhesions and tight junctions[2-5,32]. These effects induce cell rounding in cultured cells (cytopathic effect)[2-6,32,48]; (2) Arrest of the cell cycle by reduction of both the expression of cyclins and the activation of cyclin-dependent kinases that together mediate progression in the different cell cycle phases[2-4,32,48]; and (3) Cell death by apoptosis or necrosis (cytotoxic effect)[2-6,32,48]. Cell death occurs in a glucosylation-dependent/glucosylation-independent way, mainly by apoptosis with caspase-dependent or caspase-independent mechanisms[2-4,32,48]. Furthermore, the cytotoxic effects are dependent on the dose of Tcds, receptors involved, and cell types[2-6,32]. In fact, TcdB at high concentrations bind to CSPG4, FZDs, or PVRL3 and induce cell death by necrosis[36-39,59]. At low concentrations TcdB binds to CSPG4 or FZDs, and cell death occurs by apoptosis[36-39,48]. However, it must be considered that the effects of Tcds could depend on cell types, likely by selective expression of Tcd receptors, and by differential expression levels of Tcd receptors[2,3,6,32].

Tcd effect in vivo

TcdA and TcdB, *in vivo*, disrupt epithelial tight junctions and induce cell death, provoking direct damage to the colonic epithelium[2-5,32]. In addition, Tcds induce an acute inflammatory response stimulating colonic epithelial cells to release proinflammatory cytokines and chemoattractants of neutrophils[2-5,32] that can induce tissue damage, modifying the barrier effect of the intestinal mucosa. A compromised intestinal barrier within the context of active inflammation subsequently leads to enhanced intestinal and vascular permeability[3,26,29,32,34]. Following the loss of a protective barrier, there is access of Tcds and/or bacteria into the lamina propria, which in turn increases intestinal inflammation[3,26,29,32,34]. TcdA primarily affects the intestinal epithelium, while TcdB has a broader cell tropism and is probably responsible for the major clinical effects of *C. difficile* due to its toxicity, which is approximately 1000 times higher than that of TcdA. Thus, TcdB represents the main virulence factor of CDI[2,3,26,29,34]. TcdB causes death in many different cell types[3,26,29,32,34] other than epithelial cells and colonic myofibroblasts, such as hepatocytes, cardiomyocytes, lung fibroblasts, immunocytes, enteric neurons, and EGCs[32,46-49].

Since immune cells that reach the replication area of the *C. difficile* vegetative form possess intrinsic motility, the molecular strategy to cause cytoskeleton alterations and cell cycle arrest is of crucial importance in order to decrease their functional ability to counteract CDI[2,3-5,6,32]. Tcds in these immune cells stimulate cytokine secretion, in particular proinflammatory cytokines such as IFN-γ and TNF-α[2,3,6,32,34] and anti-inflammatory cytokines such as IL-10[60]. Cytoskeletal disruption occurs in some cells after 30 min, representing the initial event that leads to cell rounding in most cell types *in*

vitro[32,61] with detachment of cells. Tcds *in vivo* induces both retraction of colonocytes and basal membrane cells, favoring the additional in-depth penetration of *C. difficile* and promoting an extremely inflammatory environment that causes the expulsion of *C. difficile* in the external environment by diarrhea[32,62].

Tcds and cell death

Various Tcd-infected cells, after cell-cycle arrest, die by apoptosis[2,3,5,6,32]. This could represent an important aspect of the molecular strategy of this pathogen to reduce the inflammatory response. Apoptosis is a form of cell death that occurs without inflammation and is primarily mediated by activation of the effector caspases-3 and -7, which can be triggered by a death receptor-dependent extrinsic or a mitochondria-dependent intrinsic pathway[63-65]. However, apoptosis can also be activated in a caspase-independent manner by the cleavage/activation of pro-apoptotic Bcl-2 family members and non-caspase proteases such as calpains and cathepsins[66-69]. Caspase-dependent TcdA- and TcdB-induced apoptosis has been extensively investigated[2,3,32,48,70], while there is only one study that found that TcdA can also induce caspase-independent apoptosis following cathepsin[71] and calpain activation[71].

Recently, Fettucciari *et al*[72] demonstrated that the mechanism by which TcdB induces apoptosis is much more complex than previously thought[48,70]. TcdB induced apoptosis in EGCs, a cell population of paramount importance for colonic pathophysiology, by three signaling pathways activated by calpains, caspases, and cathepsins, which are all involved in both induction and execution of apoptotic signaling[72]. Calpain activation by Ca^{2+} influx is the first pro-apoptotic event in TcdB-induced EGC apoptosis and causes caspase-3, caspase-7, and PARP activation. The latter is activated by caspases but also directly by calpains, which are responsible for the majority of apoptosis[72]. Caspase-3/caspase-7 and PARP activation is mediated also by activation of initiator caspase-8 by TcdB, and it contributes to one-third of apoptosis events[72]. Finally, cathepsin B contributes to triggering the pro-apoptotic signal and to one-third of apoptosis events by a caspase-independent manner. It appears to control the levels of caspase-3 and caspase-7 active fragments, highlighting the complex interaction between these cysteine protease families activated during TcdB-induced apoptosis[72].

Recently, we also demonstrated that pro-inflammatory cytokines, TNF-α plus IFN-γ (CKs) strongly increased apoptosis induced by TcdB, by an enhanced activation of the three pro-apoptotic pathways induced also by TcdB alone activated by calpains, caspases, and cathepsins, which are involved in both induction and execution of apoptotic signaling[72]. However, two important differences between TcdB- and TcdB + CKs-induced apoptosis are: (1) Apoptotic signaling activation by TcdB + CKs is enriched by TNF-α-induced NF-kB signaling, inhibition of JNK activation, and activation of AKT[72]; and (2) Apoptosis induced by TcdB + CKs increased strongly in the time course, with more than 60% of the cells undergoing apoptosis at 72 h, while apoptosis induced by TcdB increased slowly, with only about 18% of cells undergoing apoptosis at 72 h[72].

This capability of TcdB to trigger three different cell death pathways represents an extremely important *C. difficile* strategy[72] to overcome the possible intrinsic resistance of a cell type to one or two of the three apoptotic pathways. In fact, as reported above, Tcds are able of cause cell death in different non-immune[32,46-49] and immune cell types[2,3-32,73,74].

A further strategy adopted by *C. difficile* is its ability to induce different types of cell death that can lead to different consequences in the *C. difficile* pathogenesis[2-4,6,32]. In fact, TcdB causes cell death by both apoptosis and necrosis[2,3,6,32,75] that is dependent on the TcdB concentration and by the TcdB receptor expressed by the target cells[20,32,36-38]. TcdB at lower concentrations and binding to the CSPG4 or FZDs receptors[20,36-40] induces apoptosis in a glucosylation-dependent manner[2-6,32], while at higher concentrations (100 pM or above) causes cell death by necrosis[20,36-43], which does not require either the autoprocessing or glucosyltransferase activities of the toxin[2-6,75]. Necrosis is an early process occurring after 2-4 h of intoxication and is found in both cell culture and colon explant models[2,3,5,-6,75]. Necrosis is characterized by quick ATP depletion, early loss of plasma membrane permeability and cellular leakage, and chromatin condensation without caspase-3 and caspase-7 activation[2,3,6,32,75].

TcdB causes necrosis through activation of a strong production of reactive oxygen species (ROS) by assembly of the NADPH oxidase complex on endosomes[2-5,75]. High levels of ROS trigger necrosis by DNA damage, lipid peroxidation, protein oxidation, and/or mitochondrial dysfunction[2,3,6,32,75]. It has been suggested that pore formation in the endocytic vacuoles is important for the glucosylation-independent necrotic cell death caused by TcdB. Indeed, a TcdB mutant, defective in pore formation, does not induce necrosis even at high nanomolar concentrations[2,4,6,75]. Unlike TcdB, TcdA does not trigger ROS production through the NADPH oxidase complex and causes glucosylation-dependent apoptosis at all concentrations tested[2-6]. The ability of TcdB, but not of TcdA, to cause necrosis may explain why a wild-type ($TcdA^+TcdB^+$) epidemic strain and an isogenic $TcdA^-TcdB^+$ mutant in animal infection models provoke considerably more damage to colon tissue than an isogenic $TcdA^+TcdB^-$ mutant strain[2-6]. The glucosylation-independent mechanism of TcdB might play a similar role in the context of human disease; TcdB-induced necrosis likely contributes to the extensive gut damage observed in patients with severe forms of CDI[2-6].

TcdB induces an early cell death defined pyknotic cell death, characterized by chromatin condensation, cell cycle arrest, and ballooning of the nuclear envelope that is both glucosyltransferase domain-dependent and -independent[2,3,6,75] and occurs at concentrations 5000 times greater than necessary for Rho protein glucosylation and ROS production[2-5,75].

It has also been reported that TcdA and TcdB trigger pyrin inflammasome activation in an apoptosis-associated speck-like protein with a caspase recruitment domain-dependent manner, causing release of IL-1β[2-6]. In particular, TcdB-induced inflammasome activation triggers a type of cell death defined as "pyroptosis," characterized by cell swelling and lysis with gross release of cellular content and inflammatory cytokines like IL-1β through pore formation in target cells caused by caspase-1-dependent activation of gasdermin D, which induces strong inflammation[2-6].

Most importantly, EGCs intoxicated with low doses of Tcds might revert to their normal functions after a brief cell cycle arrest[32,48,76], while EGCs that survive apoptotic activity of TcdB become senescent as a TcdB-mediated survival response to stressful stimulus[32,48,76]. This ability of cells surviving the cytotoxic activity of Tcds to become senescent may affect functionality of EGCs, intestinal neurons, and myocytes, contributing to altered bowel motility[32,77]. The acquisition of a senescence status by these cell types could have critical outcomes in the subsequent development of post-infectious irritable bowel syndrome and stimulation of preneoplastic cells[32,77].

Thus, we can postulate that when healing after an acute CDI patients can later have some important consequences such as a decrease in EGC number and impairment and alterations in EGC functionality [32]. After CDI, the structural and functional defects caused by the Tcds might be long-lasting in a significant percentage of patients and trigger low-grade inflammation and persistent dysmicrobism[25]. This implies that residual *C. difficile* bacteria that remain when healing after an acute CDI may benefit from this condition and provoke relapses. The latter might be due to cytotoxic synergism with inflammatory cytokines that can occur even after months without any evident cause[78]. Therefore, it is possible that *C. difficile* changes the large bowel environment to remain for a long time and favor easier relapses. This means a continual expansion of *C. difficile* carriers in the large bowel environment, with induction of an irritable bowel syndrome-like condition and with recurrences due to a latent or fluctuating inflammatory condition as in IBD subjects.

All this emphasizes the complex molecular strategy of *C. difficile* based on the cytotoxic synergism with some components of the inflammatory response as IFN-γ and TNF-α, which potentiate cytotoxicity of TcdB[48,72]. Therefore, it is conceivable that IFN-γ and TNF-α behave as drivers of the infection from the beginning of the infection, amplifying apoptosis induced by low doses of Tcds, and opening the way to infection progression[26,32,34,48,72].

Therefore, it is likely that antibiotic treatment, other than provoking dysmicrobism, by means of bacteriolytic activity builds an inflamed environment in the large bowel by release of bacterial factors from killed bacteria. Additionally, whatever inflammatory environment induced in the absence of antibiotic treatment could also help CDI in subjects with obesity or other pathologies accompanied by an inflammatory state[28].

THE CONDITIONS THAT FAVOR THE ENDEMIC SPREAD OF *C. DIFFICILE* IN PEOPLE WITH IBD

An endemic spread of *C. difficile* in IBD patients is favored by:

(1) The extreme resistance of the spores to the external environment and the mechanism of spore germination[17-19,79].

C. difficile spores are extremely resistant to strong disinfectants and radiation[17-19]. The mechanism of germination is both complicated and distinctive compared to that of other microorganisms[79], due to the peculiar Tcd interactions with the host and to cellular microbial factors that facilitate the colonization and successive infection and relapses[79-83]. Moreover, *C. difficile* spores can also adhere to gut epithelial cells and penetrate them *via* a process of macropinocytosis-like endocytosis[84,85]. This macropinocytosis-like endocytosis is dependent on Fr-95B_1 and Vn-$a_v\beta_1$ integrin and on the spore-surface collagen-like BclA3 exosporium protein[84,85]. In an *in vivo* model in mice, it has been shown that the entry of spores into intestinal epithelial cells in a dormant but reactivable state contributes to the recurrence of CDI[84,85].

(2) Progressive colonization of humans by the *C. difficile* spores depends on *C. difficile* to wait for the appropriate conditions that favor transition to the vegetative form capable of inducing infection and the clinical manifestations. Colonization is due to *C. difficile* transmission *via* the fecal-oral route, with the spores that traverse the acidic pH of the stomach to colonize the large bowel, where they remain inactive until appropriate conditions favor the passage to the vegetative form[83]. The conditions that favor intestinal germination of *C. difficile* spores are an increase of primary bile acids, butyrate, disaccharide, and trehalose and other substances produced by some bacterial species taxonomically identified in a perturbed microbiome that favors *C. difficile* overgrowth *vs* other pathogens and a reduction of secondary bile acids[86-91]. Of interest, these conditions have been described in patients with IBD[92,93]. In fact, *C. difficile* colonizes and infects the colon following antibiotic-immunosup-

pressant-induced gut dysbiosis[94-101]. The dysbiosis also depends on different factors such as age, type of drug used, administration of proton-pump inhibitors, types of foods, physical environment, the genetic and immune system of the individual, and concomitant pathologies (*e.g.*, diabetes, obesity, autoimmune and allergic diseases, IBD)[94-101]. These predisposing factors have progressively broadened the range of subjects susceptible to colonization/infection by *C. difficile*[94-101]. In turn, *C. difficile* colonization causes gut flora perturbations that increase dysmicrobism and inflammation, promoting CDI and CDI relapses[94-101]. Moreover, changes in normal gut microflora after a first CDI could predispose individuals to recurrent CDIs, and the protracted antimicrobic therapy for *C. difficile* can cause, in a gut microbiota already modified, further and persistent dysbiosis and inflammation.

Although following primary CDI episodes the bacterial taxa restore with time, in some subjects some taxa may not recover fully and maintain a decreased resistance to colonization. This in turn promotes the subsequent growth of pathogens (including *C. difficile*), altering the composition of the gut microbiome. The frequent use of antibiotics to treat *C. difficile* increases the pool of antibiotic-resistant genes in the gut microbiome, thereby favoring recurrent CDIs[96-103]. Although antibiotic exposure, hospitalization, advanced age, and immunocompromised status increase the risk for disease, community-acquired infections in otherwise healthy young and adults not previously exposed to antibiotics are not infrequent[96-103].

The favorable conditions for *C. difficile* colonization are more widespread in "developed countries" due to the increase in antibiotic therapies[15] in all ages and changes in microbiota for various external factors[104]. Thus, a progressive increase of CDI and CDI-related deaths (at present in the range of 5%-30% with primary infection)[16] is foreseeable, with a more progressive rise of death rates following CDI relapses[105,106].

(3) Persistent dysmicrobism due to the use of antibiotics, immunosuppressive drugs, and most important a colonic environment characterized by waves of inflammatory response with a continuously active basal level. Persistent dysmicrobism enables the overgrowth of several intestinal pathogens, including *C. difficile. S*ome particularities of *C. difficile* favor its growth in an altered environment characterized by low-grade inflammation[15,80-82,90,98-101,104], such as in a subject with IBD[24-31]. For primary CDI, the changes in gut microbial flora that favor overgrowth of *C. difficile* over other various intestinal pathogens (*e.g., C. perfringens*) are crucial, even though the role of the gut flora in regulating *C. difficile* is more complex than previously hypothesized. Indeed, in preventing *C. difficile* colonization, disease, and recurrence, the maintenance of enough density of the species creating an environment hostile to *C. difficile* expansion by means of both changes of biomass and composition rather than the simple reduction of some taxonomic groups plays a key role[9,90,94-101]. Furthermore, dysmicrobism depends primary on the factors and pathologies reported above (*e.g.,* IBD)[9,90,94-101].

(4) Continued emergence of new *C. difficile* strains that are more hypervirulent or multidrug-resistant (*e.g.,* ribotypes 015, 027, 078 or 176), many of which produce the binary toxin CDT[20,51,91,107,108].

(5) Production of variant Tcds by some *C. difficile* strains[51,80,91,99,102]. The expansion of CDI could be enhanced by *C. difficile* strains that produce Tcd variants[107-111]. In fact, many of these are hypervirulent and release the binary toxin CTD, which is linked with enhanced morbidity and mortality [,107-111]. CTD stimulates the formation of long cellular filaments, which become anchor points for other *C. difficile* to epithelial cells, potentiating the infection[20,51,91,107,108]. The Tcd variants are also greatly different for enzymatic activity, immunogenicity, and their receptor preference, with important implications on colonic pathology[51,91,107,108].

(6) The complex equipment of surface antigens of *C. difficile* are flagella, fimbriae, pili, cell wall proteins, and biofilm, which act as colonization factors or mediate innate immune responses that can play a key role in the persistence of *C. difficile*[2,3,6,10,32] and induce proinflammatory cytokines such as TNF-α and IFN-γ[2-6].

IMPACT OF THE LATEST KNOWLEDGE ON THE MOLECULAR PATHOGENESIS OF *C. DIFFICILE* TOXINS ON COLONIZATION/INFECTION IN INDIVIDUALS WITH IBD

The receptors for TcdA and TcdB

TcdB is the most involved in CDI due to its presence in most toxic strains and its degree of pathogenicity is 1000 times greater than that of TcdA[2-6]. The functions of the TcdB receptors, CSPG4, PVRL3, and FZD, are summarized above. The variants of TcdB are further diversified for their receptor preference with relevant implications in the pathology of CDI[32,51,91,107,108] by the ability to bind extremely different cell types[2,3,6,32], including human colon epithelium cells[43], nerve cells[47], EGCs[48], neurons, liver cells, and heart cells[49].

What are the elements that can therefore impact subjects with IBD as a consequence of the heterogeneity of Tcds and of their receptors on the various cell types? The intestinal mucosa of IBD subjects is altered by inflammation resulting from the immune response and dysmicrobism. Although direct data on how this altered colonic environment may modify the expression of the various receptors for TcdB are not available, it is likely that this could occur, as suggested by *in vitro* data. Therefore, it is possible that conditions whereby a differential expression of TcdB receptors are expressed on colonic epithelial

cells favoring primarily the necrotic effects of TcdB could arise, characterizing the trend of infection. Moreover, even the inflammatory immune cells that try to fight infections could be induced to express receptors that lead to their cell death by necrosis. Thus, in subjects with IBD an initial CDI could quickly become serious due to a strong inflammatory response that favors the expression of receptors for the TcdB that lead to death mainly by necrosis. This would profoundly change the environment already altered by necrosis to favor further relapses.

It is therefore possible that the progression and severity of CDI in subjects with IBD depends on an inflammatory environment inducing the prevalent expression of receptors that favor cell necrosis. Indeed, it has been demonstrated that expression of CSPG4 is increased by inflammatory conditions such as that induced by TNF-α and lipopolysaccharides[112,113].

Synergy of the inflammatory response and TcdB enhances the toxicity of TcdB

CKs release potentiates apoptosis of EGCs treated with low doses of TcdB[48,72]. This phenomenon is relevant with profound implications *in vivo*[26,,32,34,48,72], especially in subjects with IBD[29].

First, the enhancement of apoptosis induced by the synergism between TcdB +CKs also occurs when CKs are given to EGCs 18 h before TcdB[48]. Therefore, in an already inflamed environment, as soon as *C. difficile* begins to produce Tcds, cytotoxicity is immediately increased by the presence of pre-existing cytokines, paving the way for the progression of CDI.

Second, the cytotoxic synergism between TcdB and CKs is triggered even 3 d after infection with TcdB[48,72], implying that even if at the beginning of the infection there is no significant inflammatory response, the enhancement of cytotoxicity by cytokines can occur later.

Third, the cytotoxic synergism between TcdB + CKs at 24 h is mainly characterized by death by apoptosis[48,72], while cells surviving in the following days progressively die by apoptosis/necrosis [72]. This in contrast to the cells treated only with TcdB that die by apoptosis at 24 h, and in the following days there is only a slight increase in cell death by this mechanism[72].

Fourth, cell death induced by TcdB + CKs is characterized by the activation of three apoptotic pathways[72], with a primary role played by calpains and subsequently cathepsin B activation, which either directly or converging on the effector caspases (caspases-3 and -7) lead to cell death, bypassing any anti-apoptotic barrier in the first 24 h[72]. Thereafter, a process of amplification of the cell death process begins in the cells that have resisted apoptosis, with consequent death by apoptosis and necrosis [72]. It is therefore clear how the cytotoxic synergism between TcdB + CKs finds in subjects with IBD a particularly favorable environment that will favor CDI, characterized by a strong cell death response by necrosis, with an increase in mortality and major incidence of CDI relapses. These are due to the fact that, although partially restored with antibiotic therapy, the intestinal environment remains very susceptible to further CDI relapses for the characteristics of necrotic cell damage and the persistence of the inflammatory response.

Therefore, in subjects with IBD a circuit of progressive cell damage can be activated, which feeds on itself based on the elements that characterize the first event of colonization/infection by *C. difficile*, *i.e.* the presence of an inflammatory state that enhances the cytotoxic synergism of TcdB + CKs, with induction of apoptosis and necrosis that in turn could lead to increased expression of receptors for TcdB. The latter, based on TcdB receptors involved and TcdB concentration, will promote cell death by apoptosis and/or necrosis. Cell death causes deeper tissue damage with a progressive increase in this loop that can only be stopped with antibiotic therapy, which adds an additional level of inflammatory response. Once the infection is resolved, the environment is even more susceptible to relapses.

It is clear that if CDI occurs in a subject with IBD the clinical picture can progress to an more acute form for the following reasons: (1) The expression of high levels of receptors for TcdB that induce cell death by necrosis; and (2) Higher levels of cytokines that enhance the development of receptors for TcdB, inducing mainly necrosis. The final result is enhancement of cell death by apoptosis/necrosis.

The above considerations could explain the differences in colonic environment during CDI in individuals with IBD and therefore the susceptibility to one or more relapses[29] (Figure 1).

Perspectives for future approaches to CDI treatment

Based on some peculiarities of the pathogenesis mechanisms of *C. difficile* in subjects with IBD we can try to adopt new strategies to counteract CDI in these subjects. However, we need to bear in mind that to date efficient strategies are strongly limited for the following reasons:

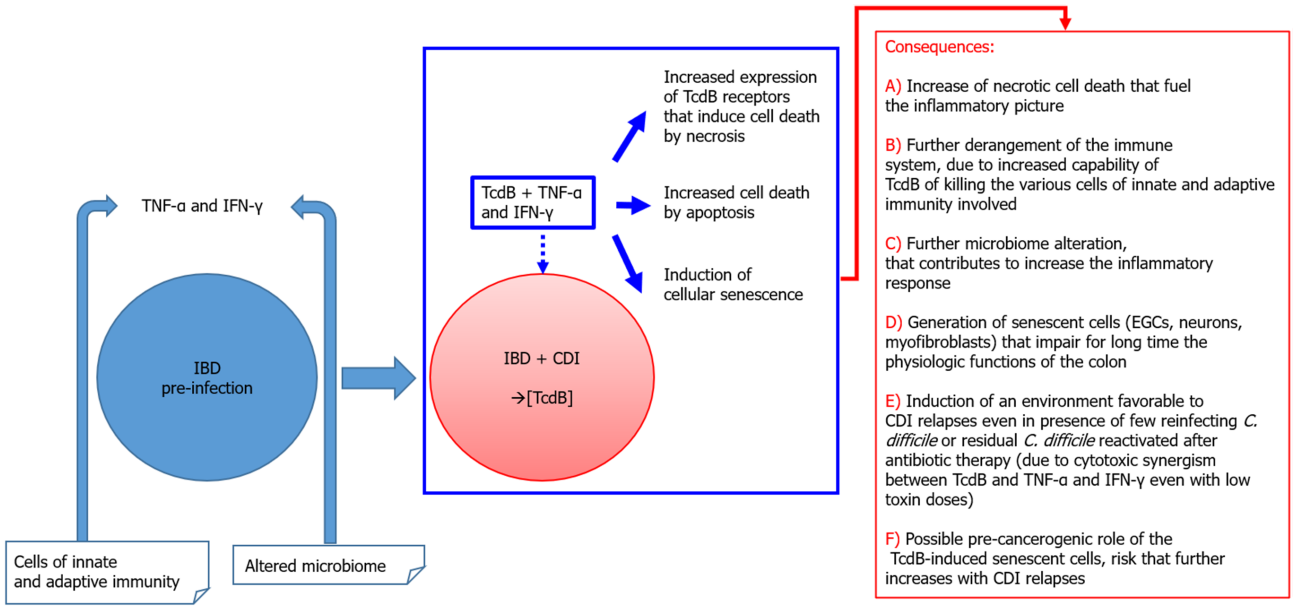
(1) It is not possible to block the endemic spread of *C. difficile* and to eradicate it in hospitals or nursing homes, which represent one of the most contaminated environments and the greatest cause of spread.

(2) Antibiotics or treatments preventing the development of conditions that favor relapses are not available.

(3) Fecal transplantation, although effective, is still a limited therapeutic.

(4) Immunotherapy with monoclonal antibodies to TcdA and TcdB has yielded very limited results.

(5) Vaccination against TcdA and TcdB did not yield significant clinical and eradication results for *C. difficile*.



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Figure 1 Causes of increased susceptibility and increased cytotoxic effect of *Clostridioides difficile* in subjects with inflammatory bowel disease. CDI: *Clostridioides difficile* infection; *C. difficile*: *Clostridioides difficile*; EGC: Enteric glial cells; IBD: Inflammatory bowel disease; IFN-γ: Interferon-gamma; TcdB: Toxin B of *Clostridioides difficile*; TNF-α: Tumor necrosis factor-alpha.

Therefore, assuming that the spread of *C. difficile* cannot be stopped and that subjects with IBD are more at risk of contracting CDI, based on the most recent knowledge on the molecular pathogenesis of *C. difficile*, which methodology of interventions can we try to develop? Here we propose several approaches: (1) Monitor subjects with IBD for *C. difficile* with greater frequency to identify the onset of the active phase of the disease as early as possible; (2) In subjects with active IBD, the presence of colonization by *C. difficile* represents a serious risk and requires a strong reduction in the inflammatory response before the infection begins or spreads; (3) In subjects already colonized with *C. difficile* and quiescent IBD, continue microbiological monitoring by evaluating the extent of colonization over time. In cases of IBD exacerbation intervene to reduce TNF-α and IFN-γ levels using appropriate targeting drugs; (4) Due to the limited availability of specific antibiotics for CDI and the continuous emergence of antibiotic resistance, the research for alternative methods is under way. For instance, pangenomic analysis of this bacterium has revealed specific drug targets toward the core genome of *C. difficile*[114]. This, in the next future, will likely pave the road for more targeted therapeutic approaches; and (5) In high-risk subjects it would be important to develop selective prophylaxis and therapy based on highly specific bacteriophages for *C. difficile*. Indeed, this is becoming a hot topic due to the impending problem of multidrug-resistant bacteria. Since it is presently possible to analyze intestinal phages, this could represent a tool of paramount importance in the future development of phage therapy[115,116]. Of interest, the feasibility of combination phage therapy to treat infections associated with IBD was recently demonstrated[117].

CONCLUSION

The increasing worldwide spread of CDI represents a serious health problem, enforced by resistance of many bacterial strains to antibiotic therapy. This is particularly worrisome for some patient groups, such as elderly institutionalized subjects, immunocompromised subjects, and subjects with IBD[118]. The latter are particularly at risk due to the basal impaired immunological status and the frequent use of antibiotics and immunosuppressant agents for treatment. It is therefore of paramount importance to understand the mechanisms favoring CDI in these patients in order to develop more targeted and effective therapeutic strategies to limit/abolish relapses, morbidity, and mortality due to this infection. It is most important to have understood that *C. difficile* to efficient colonize humans, uses key inflammatory response elements such as proinflammatory cytokines, TNF-α and IFN-γ, that favor cell death by necrosis which increases inflammation and the expression of TcdB receptors that in turn promote further necrotic cell death. It is absolutely mandatory to find prophylactic and therapeutic methodologies to antagonize this synergism between *C. difficile* and the inflammatory response. This is important to protect patients with IBD and to prevent other at-risk populations.

FOOTNOTES

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Country/Territory of origin: Italy

ORCID number: Gabrio Bassotti 0000-0002-0237-1812.

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REVIEW

Immune and metabolic cross-links in the pathogenesis of comorbid non-alcoholic fatty liver disease

Stanislav Kotlyarov

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Stanislav Kotlyarov, Department of Nursing, Ryazan State Medical University, Ryazan 390026, Russia

Corresponding author: Stanislav Kotlyarov, PhD, Researcher, Department of Nursing, Ryazan State Medical University, Vysokovoltnaya St. 9, Ryazan 390026, Russia. skmr1@yandex.ru

Abstract

In recent years, there has been a steady growth of interest in non-alcoholic fatty liver disease (NAFLD), which is associated with negative epidemiological data on the prevalence of the disease and its clinical significance. NAFLD is closely related to the metabolic syndrome and these relationships are the subject of active research. A growing body of evidence shows cross-linkages between metabolic abnormalities and the innate immune system in the development and progression of NAFLD. These links are bidirectional and largely still unclear, but a better understanding of them will improve the quality of diagnosis and management of patients. In addition, lipid metabolic disorders and the innate immune system link NAFLD with other diseases, such as atherosclerosis, which is of great clinical importance.

Key Words: Non-alcoholic fatty liver disease; Metabolism; Lipid metabolism; Lipid; Fat; Innate immune system; Pathogenesis

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Core Tip: Non-alcoholic fatty liver disease (NAFLD) is an important medical and social problem. The development of NAFLD is closely related to the metabolic syndrome, which further increases attention to the problem. The pathogenesis of NAFLD is complex and involves closely intertwined metabolic and immune mechanisms, a better understanding of which will improve the effectiveness of measures to prevent and treat the disease. Lipid metabolism has multiple connections with the innate immune system, in which various liver cells are involved.

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INTRODUCTION

Interest in non-alcoholic fatty liver disease (NAFLD) has increased significantly in recent years, due to an increasing number of reports on its high prevalence and clinical significance[1]. Epidemiologic data show that the prevalence of NAFLD in the adult population ranges from 17% to 46%, but the data vary by region and depend on age, sex, and several other characteristics[2]. These negative epidemiologic findings are thought to be related to the high prevalence of metabolic diseases, such as obesity and diabetes mellitus, which is due to the effects of low physical activity and poor diet[3]. The links of NAFLD with the metabolic syndrome are attracting increasing attention from clinicians. Dyslipidemia, obesity, insulin resistance, and diabetes are important features of the metabolic syndrome and are closely related to NAFLD[4-6]. Indeed, the prevalence of NAFLD among obese adults is 80%-90%, approximately 30%-50% in patients with diabetes, and up to 90% in patients with hyperlipidemia[7].

Another problem associated with NAFLD is that the disease is often not diagnosed in a timely manner, as patients do not seek medical care for a long time. Most patients are asymptomatic or the symptoms are nonspecific, and patients may not pay enough attention to them. In addition, these patients often have comorbidities, the clinical picture of which may be more pronounced and of greater concern to patients. Atherosclerotic cardiovascular diseases are common in these patients, significantly affecting quality of life and prognosis[8-10]. It is important to note that accurate diagnosis of NAFLD is currently associated with a number of difficulties, primarily, the limited availability of modern diagnostic tools in the primary care setting. Thus, NAFLD is currently a growing burden on patients and healthcare systems.

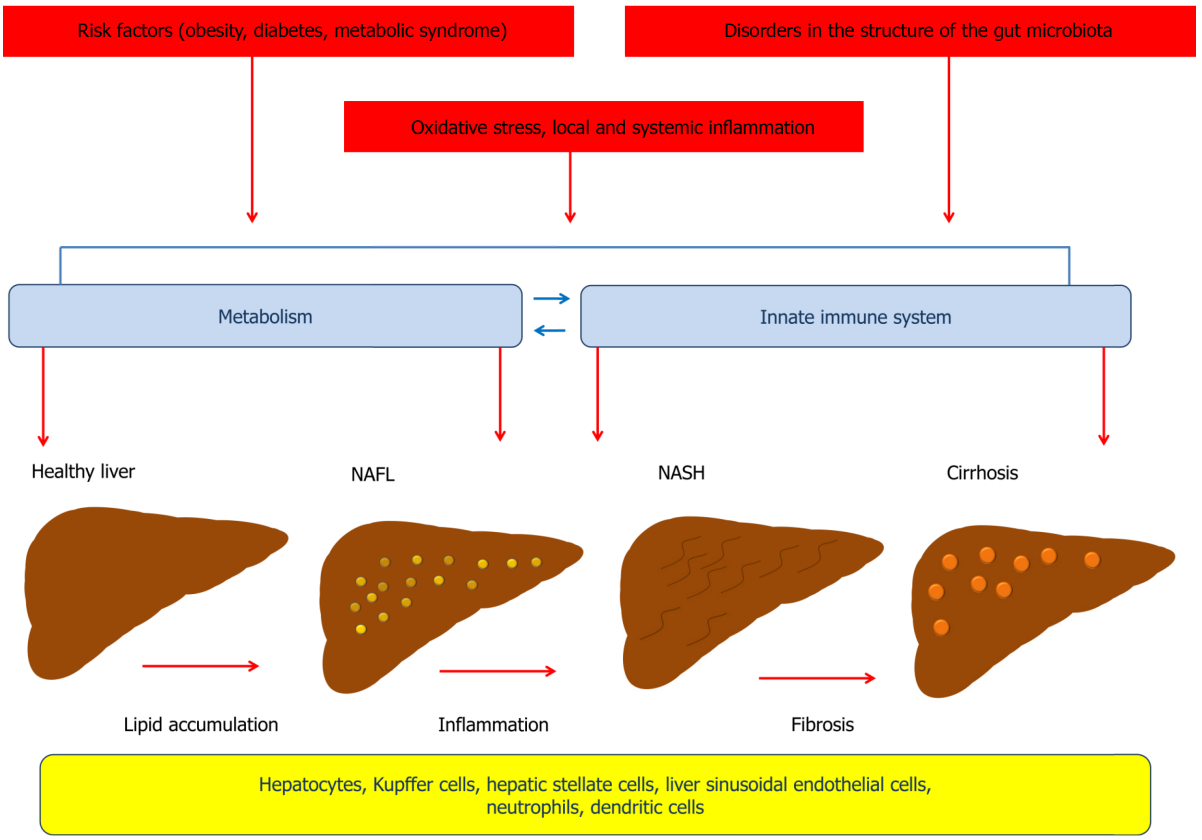
NAFLD includes two morphological forms, non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH)[11,12]. At the same time, the diagnosis of NAFLD assumes the exclusion of secondary causes and significant alcohol consumption.

NAFLD is characterized by excessive fat accumulation in the liver, but the pathophysiology of this disorder involves complex mechanisms. According to the "two-hit hypothesis" model proposed in 1998 by Day *et al*[13], the "first hit" involves lipid accumulation in hepatocytes and development of steatosis, which is associated with the negative impact of obesity, type 2 diabetes, dyslipidemia and other metabolic risk factors on the liver[13-15]. The "second hit" leads to damage to the hepatocellular system and liver inflammation and is associated with the effects of oxidative stress and proinflammatory cytokines[13]. A growing body of evidence suggests that NAFLD develops as a result of a complex chain of events, many of whose links are cross-linked, consistent with the newly proposed "multiple parallel-hit" concept. Thus, insulin resistance, de novo lipogenesis, local and systemic inflammation, disorders in the structure of the gut microbiota, and oxidative stress play an important role in the pathophysiology of NAFLD and have crosslinks that involve different cells (Figure 1)[16]. Recent advances in the study of the mechanisms that contribute to the development and progression of NAFLD have led to a better understanding of the complex interplay between environmental factors, the gut microbiota, metabolism, and the innate immune system, which include both intrahepatic and extrahepatic events[17].

MOLECULAR MECHANISMS INVOLVED IN NAFLD PROGRESSION

The significance of metabolic disorders in the pathogenesis of NAFLD

The results of studies suggest that NAFLD exhibits a close bidirectional relationship with the metabolic syndrome[18]. The development of the metabolic syndrome may precede NAFLD or be a consequence of it[19,20]. NAFLD significantly increases the risk of metabolic syndrome and may also be considered an independent risk factor for some cardiovascular diseases[21-23]. Given that NAFLD is often combined with metabolic diseases such as obesity, type 2 diabetes, hyperlipidemia, and hypertension, it may have negative prognostic implications[24,25]. Thus, an overweight person is a typical NAFLD patient phenotype[26,27]. Moreover, body mass index and NAFLD show a strong correlation[27,28]. Interestingly, NAFLD also occurs in non-obese individuals, with the majority of these findings occurring in Asian countries, although they have been described worldwide[29-32]. Despite the phenotypic differences, NAFLD patients who were not obese had similar severity of histologic liver damage[33]. At the same time, NAFLD patients without obesity had a higher degree of fibrosis[34-37].



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Figure 1 Risk factors and links of non-alcoholic fatty liver disease pathogenesis. NAFL: Non-alcoholic fatty liver; NASH: Non-alcoholic steatohepatitis.

A key histological characteristic of NAFLD is the cellular accumulation of triglyceride (triacylglycerides, TAGs) containing lipid droplets[38-40]. TAG biosynthesis is carried out using fatty acids, which may enter the cells from the blood or be formed by de novo lipogenesis and endocytotic recycling of lipoprotein remnants[40,41]. In most cases, the main source of fatty acids used for TAG formation is absorption from the blood[41,42]. Interestingly, some data suggest that TAG accumulation per se is not harmful to hepatocytes and can even be considered as a certain protective mechanism against lipotoxicity induced by free fatty acids[43]. This is supported by the data that an excess of free fatty acids in nonfat cells can lead to their dysfunction and apoptotic death[44]. Moreover, levels of free fatty acids in the blood are related to the severity of NAFLD, with saturated fatty acids being more hepatotoxic than unsaturated fatty acids[45]. Thus, free fatty acids are important mediators of excessive lipid accumulation in the liver.

Studies have shown that monounsaturated fatty acids such as oleic or palmitoleic acids are less toxic than saturated fatty acids such as palmitic or stearic acids[46,47]. Long-chain saturated palmitate induces apoptosis in Chinese hamster ovary cells through a mechanism involving reactive oxygen species (ROS) and ceramide formation, which can enhance palmitate-induced apoptosis signals[43]. In turn, unsaturated fatty acids prevent palmitate-induced apoptosis by directing palmitate to triglyceride pools and removing them from pathways leading to apoptosis[43]. In doing so, reducing the ability of cells to synthesize triglycerides contributes to lipotoxicity[43]. The mechanism of this action may be related to the fact that palmitate is poorly incorporated into cellular triglyceride pools in the absence of additional signals, but the presence of unsaturated fatty acids can help direct palmitate toward triglyceride storage, thereby excluding palmitate from apoptotic pathways. Moreover, unsaturated fatty acids, which come both as additives to the medium, such as the addition of oleate, and as a result of the action of desaturase (*e.g.*, stearoyl-CoA desaturase), demonstrate this action[43]. Stearoyl-CoA desaturase-1 (SCD), known as fatty acid desaturase, is an enzyme that is expressed in the liver and is involved in the biosynthesis of monounsaturated fatty acids, primarily oleate and palmitoleate from corresponding saturated fatty acids. Decreased expression and activity of SCD1, leads to the intake of excessive amounts of saturated fatty acids, increasing their lipotoxic effects and the development of steatohepatitis and fibrosis[48,49]. Indeed, oleic acid has been shown to be more steatogenic but has less apoptotic effects than palmitic acid in hepatocyte cell cultures[50].

Increased fat in the liver correlates directly with changes in plasma saturated fatty acids and inversely with polyunsaturated fatty acids (PUFAs)[51]. Saturated fatty acids markedly induce fat deposition in

the liver and serum ceramides, whereas dietary PUFAs prevent fat accumulation in the liver and reduce ceramides and hyperlipidemia with excess energy intake in overweight people[51]. Higher concentrations of total ω-6 PUFAs and serum linoleic acid have been shown to be associated with lower odds of developing NAFLD in the future[52]. Meanwhile, ω-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) may have a protective effect on the liver by reducing insulin resistance, reducing inflammation, and inhibiting apoptosis of hepatocytes[53].

These and other data allow us to expand our views on the features of metabolic processes in NAFLD, as well as NAFLD comorbid relationships. It has been shown that in NAFLD, regardless of the presence or absence of obesity, there is a high risk of coronary atherosclerosis, which contributes to the clinical picture[54]. It is widely known that NAFLD is associated with the development of atherosclerosis[55-57]. Moreover, NAFLD is associated with an increased risk of cardiovascular disease beyond that due to established risk factors[57]. Moreover, cardiovascular disease is the main cause of death in NAFLD patients[55].

NAFLD patients often have dyslipidemia along with other features of the metabolic syndrome. NAFLD patients have significantly elevated levels of oxidized low-density lipoprotein (LDL), and a significant association has been shown between LDL levels and the prevalence of NAFLD[58,59]. Elevated LDL levels within the normal range were associated with an increased risk of NAFLD[59]. In addition, there are important differences in LDL and high-density lipoprotein (HDL) subfractions in NAFLD patients. Liver fat has been shown to correlate more strongly with circulating HDL2 cholesterol and the ratio of HDL2 to HDL3 cholesterol than with total HDL cholesterol[60]. Patients with NASH had an increased number of small, dense LDL3 and LDL4 particles[61]. These changes may contribute to the increased risk of atherosclerosis and cardiovascular disease in these patients.

THE IMPORTANCE OF INNATE IMMUNITY IN THE PATHOGENESIS OF NAFLD

A growing body of evidence is increasing the understanding of the importance of the innate immune system in the development of NAFLD (Figure 2). The innate immune cells, which include Kupffer cells, neutrophils, dendritic cells (DCs), and natural killer (NK) cells, play an important role in the pathogenesis of NAFLD. Kupffer cells, which constitute 80% to 90% of the total macrophage population, are under physiological conditions a long-lived and self-renewing population[62]. Due to their location, they are central to innate immunity and are responsible for the rapid removal of exogenous particles such as lipopolysaccharide (LPS)[63-65]. Like other macrophages, Kupffer cells are also capable of detecting endogenous molecular signals resulting from homeostasis disruption[62].

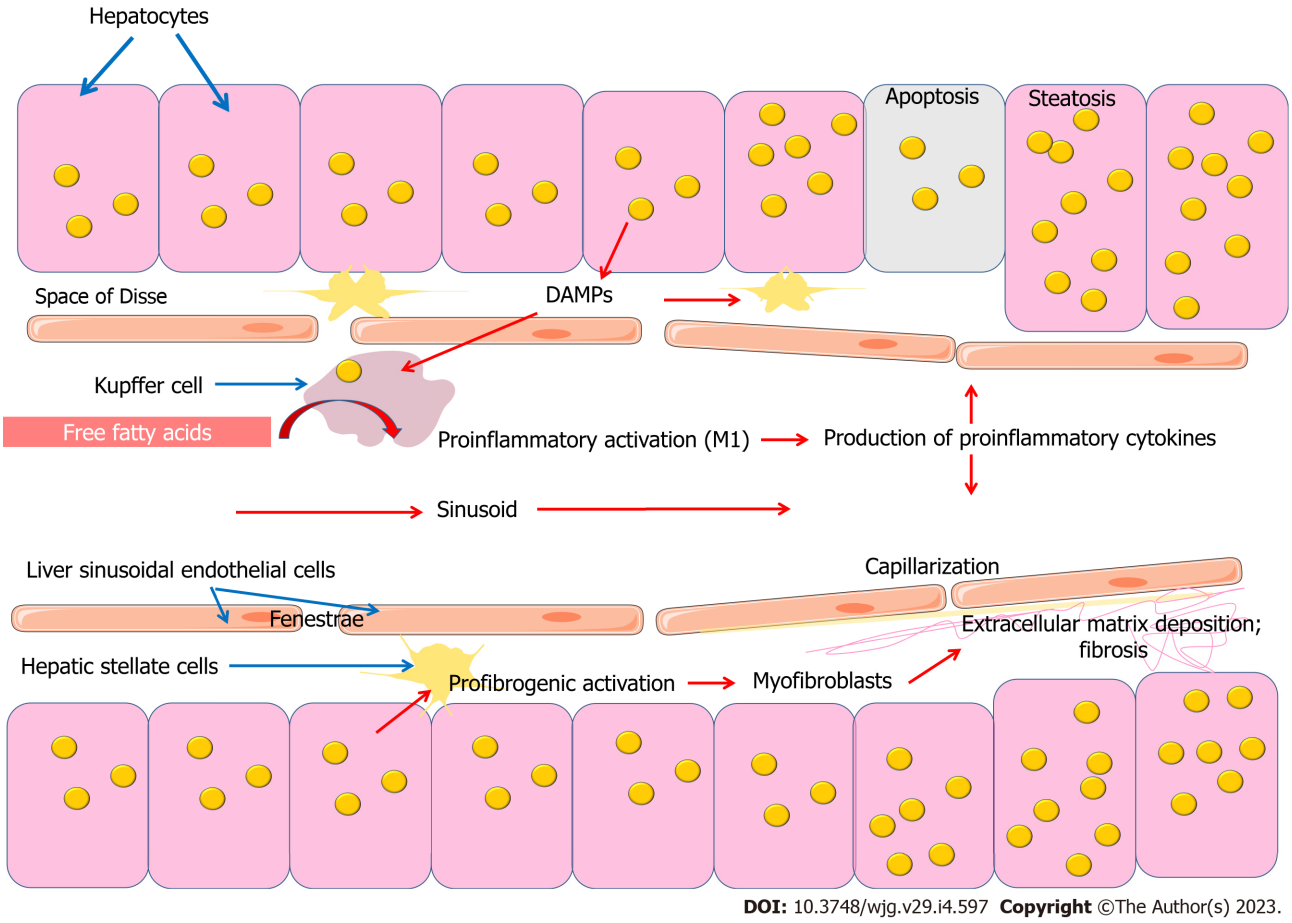
Steatohepatitis is characterized by marked enlargement and aggregation of Kupffer cells in perivenular regions, with scattered large fat vacuoles found within Kupffer cells[66]. The contribution of macrophages originating from blood monocytes to this cell pool is not entirely clear, as there is currently no marker to distinguish them from resident macrophages[67].

Kupffer cells, which are resident macrophages of the liver, uptake large amounts of free fatty acids, which contributes to their proinflammatory activation. During inflammatory activation, Kupffer cells produce proinflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, which are important participants in the progression of inflammation and development of NASH[68]. Thus, free fatty acids mediate the link between lipid metabolism and the innate immune system[69-71].

It is important to note that Kupffer cells, like other macrophages, have complex immunometabolic regulation (Figure 3). It has been shown that a prolonged high-fat diet increased the number of Kupffer cells with a proinflammatory M1 phenotype producing proinflammatory cytokines. Saturated fatty acids promoted M1 polarization of Kupffer cells, whereas ω-3 PUFAs polarized Kupffer cells to the M2 phenotype, which was associated with activation of the NF-κB and PPAR-γ signaling pathways, respectively[72]. The proinflammatory M1 phenotype of macrophages is characterized by enhanced glycolysis and fatty acid synthesis, whereas the anti-inflammatory M2 macrophages use fatty acid oxidation[73].

It has been suggested that polarization of M2 Kupffer cells may protect against fatty liver disease. M2 macrophages have been shown to be predominant in individuals with limited liver lesions, corresponding to little hepatocyte apoptosis compared with patients with more severe lesions[74]. Interestingly, M2-induced apoptosis of M1 macrophages is one of the mechanisms regulating the balance between M1 and M2 macrophages[74].

It has been suggested that elevated levels of free fatty acids, resulting from their excessive intake with food or by release from adipose tissue during starvation, may be the main cause of TNF release from Kupffer cells, leading to hepatocyte steatosis. Toll-like receptor 4 (TLR4) is able to detect free fatty acids on Kupffer cells to detect excess and overload of fatty acids in the liver[75]. It is known that saturated fatty acids can participate in the activation of TLR4, a receptor of the innate immune system[76-78]. This action can be associated with both direct stimulation of the receptor, confirming the evolutionary connection with the structure of LPS, which is the receptor aimed at detecting. In addition, fatty acids can be incorporated into the phospholipids of the plasma membrane and thus influence their biophysical properties and function[77,79]. The saturation and length of the alkyl chain are important.



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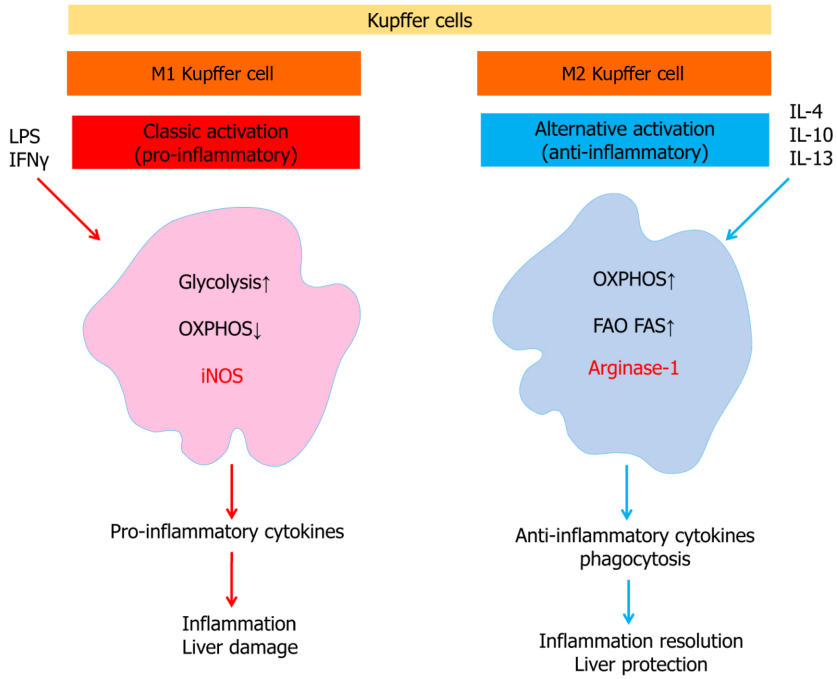
Figure 2 Cellular mechanisms of non-alcoholic fatty liver disease pathogenesis. DAMPs: Damage associated molecular patterns.

By influencing the biophysical properties of the plasma membrane and the stability of lipid rafts in this way, the function of some membrane proteins can be regulated. It is suggested that unsaturated fatty acids contribute to a decrease in lipid ordering and the stability of lipid rafts, which may lead to anti-inflammatory effects, given the role of lipid rafts as platforms for the assembly and function of many signaling pathways. Thus, unlike saturated fatty acids, unsaturated fatty acids do not have the ability to activate TLR4. In addition, their effect on the biophysical properties of plasma membranes is opposite [77].

Unsaturated fatty acids can participate in the regulation of inflammation not only due to their biophysical properties. They are also precursors for the formation of many lipid mediators associated with inflammation. The family of lipid mediators called "specialized pro-resolving mediators" includes lipoxins, resolvins, protectins and maresins. They are formed enzymatically from ω-3 and ω-6 PUFAs such as arachidonic acid, EPA and DHA. Lipoxins are formed from arachidonic acid, E-series resolvins from EPA, and D-series resolvins, protectins and maresins from DHA[80].

Circulating maresin-1 (MaR1) levels were shown to be decreased in NAFLD patients, and a negative correlation between NAFLD and serum MaR1 concentrations was found[81]. MaR1 is mainly synthesized in M2-macrophages and plays an important anti-inflammatory role. It improves insulin sensitivity and eliminates adipose tissue inflammation[82]. In addition, MaR1 improves hepatic steatosis by inhibiting endoplasmic reticulum stress and lipogenic enzymes, and inducing autophagy *via* the AMP-activated protein kinase (AMPK) pathway[81,83,84]. Activation of Kupffer cells leads to M1 polarization and a decrease in the M2 phenotype, which corresponds to a decrease in maresin production and a decrease in their anti-inflammatory effect. Resolvin D1 (RvD1), which is an endogenous mediator produced from ω-3 DHA, reduced macrophage accumulation in adipose tissue and improved insulin sensitivity in obese and diabetic mice[85]. RvD1 shifted macrophages from an M1-to-M2-like anti-inflammatory phenotype, triggering the resolution process initiated by caloric restriction in obesity-induced steatohepatitis[86]. Protectin DX, derived from DHA, showed suppressive effects on inflammation and insulin resistance and improved hepatic steatosis by suppressing endoplasmic reticulum stress through AMPK-induced ORP150 expression[87].

On the other hand, the development of NAFLD correlates with an increase in serum eicosanoids. Moreover, profiling of plasma eicosanoids and other PUFA metabolites can differentiate NAFLD from NASH[88]. 11,12-dihydroxy-eicosatrienoic acid (11,12-diHETrE) was used as a biomarker to differ-



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Figure 3 Kupffer cell polarization. LPS: Lipopolysaccharide; FAO: Fatty acid oxidation; FAS: Fatty acid synthesis; IFN-γ: Interferon gamma; IL: Interleukin; iNOS: Inducible nitric oxide synthase; OXPHOS: Oxidative phosphorylation.

entiate NAFLD from NASH[88]. In another study, patients with NASH had significantly elevated levels of 9- and 13-HODE and 9- and 13-oxoODE, products of linoleic acid oxidation, compared with patients with steatosis[89]. Interestingly, patients with stage I NAFLD had lower plasma levels of 5-HETE, whereas patients with stage II steatosis had higher concentrations of 9-HODE[90].

Thus, lipid metabolites derived from fatty acids are involved in the development of NAFLD, which is an interesting topic for further research (Figure 2).

Hepatocellular accumulation of lipids can modulate the biological activity of Kupffer cells through a number of mechanisms. On the one hand, fat-saturated hepatocyte swelling changes the architecture of the sinusoidal network, reducing intrasinusoidal volume and microvascular blood flow. Disruption of microvascular blood flow also contributes to the involvement of sinusoidal endothelial cells, Kupffer cells, stellate cells and involvement of inflammatory cells and platelets[91]. Later developing fibrosing steatohepatitis with capillarization of the sinusoids, increases narrowing and distortion of the sinusoidal lumen, further limiting microvascular blood flow. In addition, leukocytes entering the narrowed sinusoids may adhere to the endothelium as a result of activation of the hepatic microvascular inflammatory response[91]. On the other hand, fat overload of hepatocytes causes lipotoxicity and the release of damage-associated molecular patterns (DAMPs), which can activate Kupffer cells and hepatic stellate cells (HSCs), promoting inflammation and fibrosis[92]. Lipid accumulation in hepatocytes has been shown to induce the release of factors that accelerate the activation and proliferation of HSCs and increase their resistance to apoptosis[93]. Conditioned medium from steatotic hepatocytes induced expression of the profibrogenic genes transforming growth factor (TGF)-beta, tissue inhibitor of metalloproteinase-1 (TIMP-1), TIMP-2 and matrix metalloproteinase-2, and expression of the NF-κB-dependent monocyte chemotactic protein-1 (MCP-1) in HSCs[93]. Thus, quiescent HSCs participate in the maintenance of liver architecture by maintaining the balance of extracellular matrix, while disruption of this balance, for example, due to metabolic disorders, leads to HSCs activation and fibrosis[94,95].

Hepatocytes exposed to apoptosis form apoptotic bodies, which are phagocytosed by HSCs and Kupffer cells, triggering a profibrogenic response due to transdifferentiation of HSCs into collagen-producing myofibroblasts[96]. Apoptotic cell uptake has been shown to stimulate Kupffer cell production of death ligands, including Fas ligand and TNF-alpha, which promotes inflammation and fibrogenesis[97].

An important pathogenetic mechanism involved in the pathogenesis of NAFLD is the role of the intestinal microbiota and a defect in the intestinal barrier caused by liver damage. Impaired gut barrier function is thought to accelerate translocation of enteric LPS, which activates proinflammatory signaling pathways and the release of related inflammatory factors in the liver[98]. Intestinal bacterial microflora and TLR4 have been shown to be involved in liver fibrogenesis[99]. *Escherichia coli* LPS can enhance liver damage in NAFLD by inducing macrophage and platelet activation through the TLR4 pathway [100]. Plasma endotoxin levels and inflammatory markers have been shown to be significantly higher in NAFLD compared with controls and to increase with the severity of hepatic steatosis[101]. Proinflam-

matory activity and immune imbalance associated with the pathophysiology of NAFLD may be related to gut dysbiosis[102]. For example, decreased Bacteroidetes and increased Firmicutes were observed in obese individuals[102]. Changes in gut microflora ratios may also increase endogenous ethanol production, which generally increases gut permeability, and contributes to translocation of endotoxins from the gut lumen into the portal bloodstream[102,103].

Another immunometabolic link between the gut microbiota and NAFLD, related to short-chain fatty acids (SCFAs), should also be noted[104,105]. SCFAs are formed by the gut microbiota during the fermentation of non-digestible fibers such as resistant starch, cellulose, and pectin[106]. SCFAs are used by colonic mucosal epithelial cells as an energy substrate, are involved in the regulation of a number of processes in the intestinal wall or enter the portal bloodstream, and may be involved in the formation of immunometabolic connections with other organs[107].

A growing body of evidence strengthens the understanding of the importance of SCFAs in inflammation. SCFAs act *via* receptors associated with the G-protein GPR43 and GPR41, also known as free fatty acid receptor (FFA)2 and FFA3, respectively[108-111]. In addition, SCFAs realize their action through inhibition of histone deacetylase (HDAC)[112,113].

Butyrate is well known for its anti-inflammatory properties and is of great clinical interest[107,114,115]. Through HDAC3 inhibition, butyrate can induce a metabolic switch of macrophages toward an anti-inflammatory M2 phenotype[112,113].

SCFAs are also known to affect the differentiation, recruitment and activation of neutrophils, DCs, macrophages and monocytes as well as T cells[116,117]. Butyrate is involved in the regulation of DC differentiation derived from human monocytes, keeping DCs in the immature stage[118].

In addition to their involvement in inflammation, SCFAs regulate lipid metabolism in the liver. Butyrate levels have been shown to decrease in NAFLD patients and mice with decreased estrogen levels, with butyrate administration attenuating liver steatosis[119]. Studies in rats fed a high-fat diet (HFD) have shown that butyrate increases β-oxidation of fatty acids, inhibits lipid synthesis and suppresses nuclear factor-kappa B and inflammation[120,121]. The addition of sodium butyrate protects mice from developing NASH. It is important to note that the metabolic role of SCFAs in liver function is rather complex[122]. In addition to attenuating hepatic steatosis, acetate, another SCFA derived from the microbiota, may conversely promote hepatic lipogenesis after excessive fructose intake[123,124].

A growing body of evidence strengthens the understanding that lipoproteins are part of an important transport mechanism that is utilized by the innate immune system. The mechanism of LPS elimination involves LPS disaggregation and binding to circulating lipoproteins, uptake of lipoprotein-associated LPS by the liver, and excretion of LPS with the bile[125,126]. This pathway, known as reverse LPS transport, involves lipoproteins as the main carriers of LPS in the plasma and includes the proteins LBP, BPI, phospholipid-transfer protein (PLTP), and cholesteryl ester transfer protein (CETP), which belong to the lipid transfer/LPS binding gene family (LT/LBP) and play different roles in LPS metabolism [126]. In addition, reverse cholesterol transport is at the beginning of the cross-talk between cholesterol metabolism and the innate immune system[126]. ABCA1, a key participant in reverse cholesterol transport also contributes to the efflux of LPS from macrophages[127]. HDL and other plasma lipoproteins have been shown to contribute to the release of LPS from the cell surface of monocytes[128].

Lipid transfer proteins (lecithin-cholesterol acyltransferase (LCAT), CETP, and PLTP) as well as hepatic and endothelial lipases remodel HDL in the bloodstream. CETP is part of a family of proteins including LPS-binding protein (LBP) and bactericidal permeability increasing protein (BPI) and may participate in the transport of LPS between lipoproteins for further utilization in the liver. CETP transports cholesterol esters from HDL to apoB-containing LDL and very low density lipoproteins (VLDLs).

Kupffer cells take up most of the LPS and can inactivate it by deacylation with acyloxyacyl hydrolase. Kupffer cells express high levels of class A scavenger receptors (SR-A), which bind oxidized low-density lipoproteins (LDL) and are also involved in LPS uptake[64,129]. SR-A expression is increased by oxidized LDL[130,131]. Importantly, in the liver, SR-A is also important for cell adhesion, suggesting a role for SR-A in the recruitment and retention of cells in various organs or in sites of pathological conditions, such as foci of inflammation or areas of atherosclerotic lesions[64]. In addition to Kupffer cells, SR-A types I and II are expressed in the liver on endothelial cells, which are less able to bind LPS [132].

Interestingly, plasma CETP predominantly originates from Kupffer cells, and plasma CETP levels predict the content of Kupffer cells in the liver in humans[133]. In addition, activation of Kupffer cells by LPS strongly decreases CETP expression[134]. LPS has been shown to activate resting Kupffer cells, resulting in decreased hepatic CETP expression and decreased CETP in plasma and increased HDL cholesterol levels[135]. Importantly, CETP inhibition improves HDL function but leads to liver obesity and insulin resistance in CETP-expressing transgenic mice on a HFD[136]. Information obtained in recent years has improved the understanding of the role of CETP in inflammation. Experimental evidence suggests that CETP in macrophages as well as in the liver prevents LPS interaction with TLR4, thereby reducing the inflammatory response[137]. Compared with wild-type mice, CETP mice showed a higher survival rate after polymicrobial sepsis. CETP mice had lower plasma IL-6 concentrations and decreased levels of hepatic TLR4 and acyloxyacyl hydrolase protein[137]. Species-specific differences in CETP expression should be noted[138]. In mice and rats, in contrast to humans, as well as primates,

rabbits, and hamsters, CETP is absent in plasma. Consequently, wild-type mice, have naturally low LDL and high HDL levels, in which up to 90% of cholesterol is transported and have low susceptibility to developing atherosclerosis. Transgenic mice expressing human CETP have increased reverse cholesterol transport, which is associated with increased clearance of apoB lipoproteins in the liver. They also show increased postprandial triglyceridemia, increased liver uptake of LPS, and increased survival in endotoxemia[139,140]. Transgenic expression of CETP in mice also reduces liver fat accumulation and improves insulin sensitivity in diet-induced obesity[141,142]. CETP has been shown to reduce liver TAG content in female mice through enhanced β-oxidation and to promote the synthesis and assembly of VLDL[142]. CETP inhibition in transgenic CETP-expressing mice disrupted TAG metabolic pathways, leading to liver TG accumulation and insulin resistance in diet-induced obese mice[136]. In addition, CETP inhibition by anacetrapib increased systemic and hepatic inflammation to a greater extent in obese mice[136].

Despite its weaker ability to bind LPS compared to LBP or BPI, CETP is associated with resistance to sepsis. Experiments with human CETP transgenic mice showed lower mortality after LPS administration compared to wild-type mice. The pathway involving CETP is of interest because it represents a cross-talk mechanism of reverse cholesterol transport and the innate immune system, in which LPS and cholesterol share common transport and utilization pathways[140].

Neutrophils, other important participants in the innate immune system, are also involved in the pathogenesis of NAFLD[143]. Given that inflammation is a key event that contributes to the progression of fatty liver dystrophy to NAFLD, these patients show significant neutrophil infiltration into the liver, often accompanied by increased expression of chemokines that promote neutrophil chemotaxis[144].

Neutrophils exhibit cross-links with HSCs. On the one hand, neutrophils activate HSCs through the production of ROS[145-147]. On the other hand, activated HSCs have been shown to support neutrophil survival by producing granulocyte-macrophage colony-stimulating factor and IL-15. This may serve as a positive direct loop contributor to liver damage and fibrosis under a HFD[147].

Interestingly, it has been shown that neutrophils in blood in patients with NASH had increased expression of receptors reflecting the preparation of neutrophils to migrate into tissue. In addition to preparation for migration, blood neutrophils in NASH were also functionally activated[148]. They were characterized by increased IL-8 production and had more than double the spontaneous oxidative burst. In analyzing these data, it was noted that neutrophils can not only move from the vascular lumen into extravascular tissues but can also move back into the bloodstream, through a process known as reverse transendothelial migration. Reverse transendothelial migration is of interest due to its possible interaction with the immune system[149]. However, its possible role in NAFLD has yet to be studied.

Thus, neutrophils play an important role in the development of inflammation and liver fibrosis[150]. On the other hand, neutrophils contribute to the spontaneous resolution of inflammation and liver fibrosis. Acting *via* miR-223, neutrophils act as resolving effector cells that induce the transition of proinflammatory macrophages to a restorative phenotype by suppressing NLRP3 inflammasome expression[151]. Another study in a diet-induced NASH mouse model also showed a phase-dependent contrasting role of neutrophils as triggers and pro-resolutive mediators of liver injury and fibrosis[150]. In addition to these findings, miR-223 was shown to be elevated in hepatocytes from HFD-treated mice and patients with NASH, which may be due to the fact that miR-223 can be transferred from neutrophils *via* the exosome. Moreover, miR-223 in hepatocytes acts as an anti-inflammatory molecule, directly affecting several inflammatory genes[152].

Thus, neutrophils play a complex multifaceted role in the pathogenesis of NAFLD, which is a promising topic for further research.

Liver DCs are a heterogeneous population of hepatic sinusoidal antigen-presenting cells[153,154]. DCs exist in mature or immature states and undergo maturation when exposed to immune or inflammatory signals such as microbial products and proinflammatory cytokines. DCs are involved in maintaining immune homeostasis and liver tolerance by promoting CD8+ T-cell elimination, as well as secreting anti-inflammatory cytokines that maintain the quiescent HSC state and promote TLR4 refractoriness to LPS. In addition, DCs regulate the number and activity of cells involved in the development of fibrosis and may play a role in the regression of liver fibrosis[155]. Dendritic cells can contribute to liver fibrosis regression by activating metalloproteinases and contribute to the homeostasis of NK cells, which are mainly antifibrogenic[154].

Natural killer cells are a heterogeneous multifunctional population of lymphoid cells located inside the sinusoidal space, where they can attach to endothelium and Kupffer cells[156]. A key factor determining the activity of these cells in NASH is their metabolic reprogramming.

Liver NK cells are part of the innate immune system and may play an important role in NAFLD. However, the regulation and function of NK cells in NAFLD remains controversial due to their different involvement at different stages of the disease. On the one hand, NK cells are active and may be useful in the early stages of fibrosis, when they contribute to TRAIL-mediated HSC death. On the other hand, NK cell involvement becomes detrimental when they lose their antitumor capacity, which may contribute to disease progression in later stages[156]. Indeed, metabolic reprogramming of NK cells in obesity limits the antitumor response, which is known as "metabolic paralysis"[157]. Overload of NK cells with lipids absorbed from the environment in obesity leads to metabolic defects that cause inhibition of the cytotoxic mechanism, resulting in loss of antitumor functions[157]. Overall, the available data suggest a

possible therapeutic potential for the regulation of NK cell function, which is a promising topic for further research.

ROLE OF RECEPTORS IN THE INNATE IMMUNE SYSTEM

The innate immune system relies on a large number of pattern recognition receptors (PRRs) to recognize both DAMPs and pathogen-associated molecular patterns. Toll-like receptors (TLRs) are the most well characterized representatives of PRRs. They are expressed in a variety of liver cells, including Kupffer cells, HSCs, hepatocytes, sinusoidal endothelial cells, and biliary epithelial cells[158-160]. A growing body of evidence reinforces the importance of TLRs in the pathogenesis of NAFLD[161]. TLR4 is of particular interest in connection with liver inflammation and fibrogenesis[158,162,163]. TLR4 is a receptor that detects the LPS of Gram-negative bacteria and is widely known for its role in various diseases.

TLR4 is expressed on all types of liver cells, including Kupffer cells, HSCs, and hepatocytes. Under normal conditions, hepatic cells express minimal TLRs, indicating a high tolerance of the liver to TLR ligands[164]. At the same time, receptor expression in the liver is associated with inflammation and fibrosis[164]. TLR4 plays a central role in Kupffer cell activation by responding to LPS. LPS is considered a potent inducer of hepatic inflammation. It promotes the production of TNF-α in Kupffer cells, which is a mediator of inflammation in the pathogenesis of NAFLD[165]. In addition, LPS can activate HSCs, and Kupffer cells can enhance this process by producing TGF-β and making HSCs more sensitive to TGF-β[164]. Despite the fact that Kupffer cells are the main targets for LPS in the liver, it is HSCs that contribute to TLR4-dependent fibrosis[99]. In addition, modulation of TGF-β signaling along the TLR4-MyD88-NF-κB axis provides a link between proinflammatory and profibrogenic signals[99]. Numerous data support the involvement of HSCs as central mediators of hepatic fibrosis. Activation of TLR4 in quiescent HSCs enhances chemokine secretion and induces Kupffer cell chemotaxis and inhibits the TGF-β pseudoreceptor Bambi, which increases HSCs sensitivity to signals induced by TGF-β and enables unrestricted activation by Kupffer cells[99]. A significantly reduced expression of the Bambi gene in HSCs was seen when incubated with the TLR4 LPS ligand[166].

It was found that a diet high in cholesterol leads to the accumulation of free cholesterol in HSCs, which promotes TLR4 signaling by increasing TLR4 levels in the membrane and can suppress Bambi gene expression. As a consequence, TGF-β signaling in HSCs was enhanced, leading to HSCs activation and progression of liver fibrosis[166].

ENDOTHELIAL CELL INVOLVEMENT IN THE IMMUNE SYSTEM IN THE LIVER

Endothelial cells, which form the inner membrane of blood vessels, play an important role in the functioning of the barrier between blood and tissues. Endothelium is characterized by heterogeneity and plasticity due to phenotypic specialization of different tissue types. This endothelial specialization can provide dense connections necessary for functioning of histo-tissue barriers, or on the contrary can promote infiltration and extravasation of molecules and particles circulating in the bloodstream due to fenestrated endothelium in the liver and kidneys[167]. Given that the liver is a highly vascularized organ (accounting for 20% of cardiac output), hepatic sinusoidal endothelial cells constitute a significant proportion of the total number of liver cells[168]. Liver sinusoidal endothelial cells (LSECs) have a unique morphological phenotype characterized by a combination of numerous fenestrae and lack of a basement membrane, which provides open access for dissolved substances between the sinusoidal blood and the Disse space (Figure 2). LSECs are involved in regulation of the liver microenvironment and act as the liver's first protective barrier. An important functional phenotypic feature of hepatic sinusoidal endothelial cells is their high endocytic capacity[169]. These cells are capable of absorbing and removing soluble macromolecules from the portal venous blood in addition to Kupffer cells located on the lumen side of the endothelium[168].

Disruption of the LSECs phenotype is a critical step in the liver fibrosis process (Figure 2). Capillarization, in which there is a lack of fenestration of hepatic sinusoidal endothelial cells and formation of an organized basal membrane, precedes fibrosis and contributes to HSC activation[169]. Vascular endothelial growth factor (VEGF) produced by hepatocytes and HSCs has been shown to be a key regulator of the LSEC phenotype[169-173]. The maintenance of the fenestrated LSEC phenotype is provided by the action of VEGF through a nitric oxide (NO)-dependent and NO-independent pathway [169-171]. In this case, VEGF, which is produced by hepatocytes or stellate cells, promotes NO formation from LSECs *via* endothelial nitric oxide synthase (eNOS)[171].

A growing body of evidence supports the important role of the endothelium in vascular biology. Endothelial cells can detect changes in blood flow and are involved in the regulation of hemodynamics and inflammation through the production of several bioactive substances. Endothelial production of NO is the best known way to regulate vascular hemodynamics. Nitric oxide is an important signaling molecule that is at the crossroads between the regulation of vascular hemodynamics and innate

immunity[174]. Importantly, NO demonstrates active involvement in the regulation of inflammation in the vascular wall, which is important in the development of atherosclerosis. Endothelial NO actively regulates the innate immune response involved in atherogenesis by regulating macrophage and lymphocyte uptake and vessel wall migration *via* adhesion molecules[174].

Nitric oxide synthesis in the endothelium is carried out by a specific constitutive eNOS isoform. Mechanical stimulation of endothelial cells by blood flow triggers a complex chain of events involving numerous cellular mechanosensors and enzymes, leading to activation of eNOS[175]. eNOS is expressed in LSECs and produces small amounts of NO, which maintain intrahepatic sinusoidal vascular tone and hemodynamics in the liver. Another isoform of nitric oxide synthase, inducible NOS (iNOS) is expressed in various liver cells, including LSECs, hepatocytes, Kupffer cells, HSCs and other immune cells[176-178]. LPS induces iNOS expression and NO production and increases caveolin-1 and decreases eNOS phosphorylation[179].

It should be noted that while the NO produced by eNOS has a hepatoprotective effect by inhibiting inflammatory activation of Kupffer cells, the NO produced by iNOS, in contrast, promotes NAFLD [180]. iNOS produces significantly more NO than eNOS, which can have negative effects. This is due to the cytotoxicity of NO in high concentrations. In particular, peroxynitrite (ONOO-) can damage a wide range of cellular molecules[181]. Interestingly, peroxynitrite can affect cyclooxygenase (COX)-1 and COX-2 activity depending on the concentration[182,183]. It has been suggested that NO can interact directly with COX, for example *via* S-nitrosylation, causing an increase in its enzymatic activity[184, 185]. Thus, NO production has closely overlapping connections with innate immunity. These and other data suggested a role for COX enzymes as important endogenous receptor targets for NO functions [186]. COX-2-mediated inflammation is important for insulin resistance associated with obesity and fatty liver dystrophy. Daily aspirin intake was associated with less severe histologic signs of NAFLD and NASH and a reduced risk of fibrosis progression over time[187].

Importantly, eNOS activity is decreased in pathological conditions, whereas iNOS activity is increased. Decreased NO production in LSECs causes endothelial cell capillarization and HSCs activation. This leads to deposition of extracellular matrix, proliferation of HSCs, increased intrahepatic resistance and impaired sinusoidal blood flow[180].

Thus, the function of NO is related to the maintenance of liver cell function. NO derived from eNOS protects against liver disease, whereas NO derived from iNOS has a proinflammatory effect[180]. When mice were fed a HFD, a decrease in liver NO was shown to precede the onset of liver inflammation through the NF-κB pathway as well as impaired insulin signaling at the IRS-1 and phospho-Akt levels. Thus, an important physiological role of endothelial NO has been shown to limit obesity-associated inflammation and impaired insulin signaling in hepatocytes and Kupffer cells *via* the NO/cGMP-dependent protein kinase (PKG)/ vasodilator-stimulated phosphoprotein (VASP) pathway as part of a cross-talk mechanism with metabolic disturbances associated with obesity[168].

LSECs exhibit a proinflammatory phenotype during the progression of NAFLD to NASH. It is characterized by surface overexpression of adhesion molecules such as ICAM-1, VCAM-1, and VAP-1 (AOC3) and production of proinflammatory molecules such as TNF-α, IL-6, IL-1, and MCP1 (CCL2)[188,189]. Interestingly, LSECs and HSCs are involved in maintaining each other's differential phenotype. On the one hand, VEGF-A production by either HSCs or hepatocytes supports LSECs differentiation[170]; on the other hand, fenestrated LSECs prevent HSCs activation and promote the conversion of activated HSCs to a dormant state. However, LSECs lose this effect when they are undifferentiated or have a capillarized phenotype[171,190].

Thus, LSECs play an important role in liver immunology and the development of NAFLD. In contrast to hepatocytes, free fatty acids such as palmitic acid and oleic acid inhibit LPS-induced production of proinflammatory chemokines in LSECs and inhibit inflammatory cell recruitment. These findings suggest a potentially protective role for LSECs in the liver with excess free fatty acids, as in NAFLD [191].

A growing body of evidence suggests that the role of lipid metabolism in endothelial cell function is not only as a structural or energetic substrate, but also as a participant in cell mechanobiology. In doing so, lipids are at the intersection of chemo- and mechanobiological signaling pathways.

CONCLUSION

NAFLD is a widespread disease whose clinical and pathophysiological links are only beginning to be understood. TAG accumulation in hepatocytes in NAFLD results from a complex chain of events and is complicated in nature, involving many exogenous and endogenous factors. Obesity and impaired lipid metabolism are considered to be the key links in the development of NAFLD. Moreover, impaired fatty acid metabolism is one of the central events in the pathogenesis of NAFLD due to their involvement not only as an energy substrate or their structural function in cells, but also due to their connection with the innate immune system. Lipid metabolism has multiple cross-links with the innate immune system, and these links are important in the pathogenesis of NAFLD.

Analysis of the data allows us to emphasize the need for a better study of the multifaceted role of lipid metabolism and its disorders as a link in the complex chain of processes underlying the development of NAFLD.

The pathogenesis of NAFLD is an important target for further research, among which immunometabolic cross-linkages can be considered as one of the promising directions. Immunometabolic regulation of cells and intercellular connections at different stages of liver disease development can be a significant target for therapeutic intervention. In addition, the immune and metabolic axes that link the liver to other organs are also of research and clinical interest. There is a growing understanding that the gut microbiota is an important participant in immune and metabolic processes not only in the gut, but also in other organs. There is also interest in information on the cross-linkages of lipid-transport function and innate immunity, which have evolutionarily conservative roots and link a number of diseases that mutually influence their natural history.

In summary, NAFLD is a complex multifaceted disease whose keys are still unknown to clinicians and researchers, but a better understanding of metabolic and immune cross-linkages will improve patient diagnosis and treatment approaches.

FOOTNOTES

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ORCID number: Stanislav Kotlyarov 0000-0002-7083-2692.

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REVIEW

Iron as a therapeutic target in chronic liver disease

Elias Kouroumalis, Ioannis Tsomidis, Argyro Voumvouraki

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Elias Kouroumalis, Liver Research Laboratory, University of Crete Medical School, Heraklion 71003, Greece

Ioannis Tsomidis, Argyro Voumvouraki, First Department of Internal Medicine, AHEPA University Hospital, Thessaloniki 54621, Greece

Corresponding author: Elias Kouroumalis, MD, PhD, Emeritus Professor, Liver Research Laboratory, University of Crete Medical School, 13 Kalokerinou Street, Voutes, Heraklion 71003, Greece. kouroumi@uoc.gr

Abstract

It was clearly realized more than 50 years ago that iron deposition in the liver may be a critical factor in the development and progression of liver disease. The recent clarification of ferroptosis as a specific form of regulated hepatocyte death different from apoptosis and the description of ferritinophagy as a specific variation of autophagy prompted detailed investigations on the association of iron and the liver. In this review, we will present a brief discussion of iron absorption and handling by the liver with emphasis on the role of liver macrophages and the significance of the iron regulators hepcidin, transferrin, and ferritin in iron homeostasis. The regulation of ferroptosis by endogenous and exogenous modulators will be examined. Furthermore, the involvement of iron and ferroptosis in various liver diseases including alcoholic and non-alcoholic liver disease, chronic hepatitis B and C, liver fibrosis, and hepatocellular carcinoma (HCC) will be analyzed. Finally, experimental and clinical results following interventions to reduce iron deposition and the promising manipulation of ferroptosis will be presented. Most liver diseases will be benefited by ferroptosis inhibition using exogenous inhibitors with the notable exception of HCC, where induction of ferroptosis is the desired effect. Current evidence mostly stems from *in vitro* and *in vivo* experimental studies and the need for well-designed future clinical trials is warranted.

Key Words: Iron overload; Liver disease; Ferroptosis; Ferritinophagy; Ferroptosis modulators

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Core Tip: Iron overload may damage the liver in a variety of liver diseases such as cirrhosis and hepatocellular carcinoma affecting patient survival. In this review, we present the evidence, both experimental and clinical, of the detrimental effects of iron deposition in hepatocytes and other liver sinusoidal cells. Moreover, we examine the mechanism and implications of the recently described ferroptosis in the evolution of liver disease. Ferroptosis is a form of regulated hepatocyte death caused by excess iron and lipid peroxidation. Inhibition or induction of ferroptosis may profoundly improve the course of many liver diseases as demonstrated by a large number of experimental studies as well as a small number of clinical trials.

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INTRODUCTION

The major suppliers of plasma iron are duodenal enterocytes and iron-recycling macrophages[1-3]. Duodenal cytochrome B reductase reduces inorganic trivalent iron reaching the duodenum to form divalent iron (Fe^{2+}), and surface divalent metal transporter 1 (DMT1) imports Fe^{2+} into the cytoplasm. The gene *SLC11A2* encoding DMT1 is activated in cases of iron deficiency or hypoxia as it interacts with the hypoxia-inducible factors (HIF1α and HIF2α) overexpressed in these situations[4-7]. The cytoplasmic iron sensor iron-responsive element (IRE) and iron regulatory proteins (IRP1 and IRP2) also participate in iron absorption control as they stabilize the *SLC11A2* transcript in iron deficiency or dissociate and degrade in iron overload[8]. Then, the cytoplasmic divalent iron is bound to ferroportin, the only known iron exporter protein, and exported to the portal vein blood. Transportation is mediated by the chaperone protein poly (rC)-binding protein 2 encoded by the *SLC40A* gene[9]. The main regulator of ferroportin is hepcidin[10], but the IRP/IRE proteins and microRNAs are also involved[11]. Once in the portal vein, the divalent iron is oxidized back to trivalent by the ferroxidases hephestin and ceruloplasmin and then carried in different cells bound to transferrin. Peripheral cells import iron by the internalization of transferrin after it binds to its receptor TFR1[12] and is sorted into endosomes where iron is removed in the acidic environment, reduced again to $Fe2^{+}$ by the ferrireductase STEAP3, and released into the cytosol by DMT1[1,3]. Iron is then either exported by ferroportin or stored in ferritin or in the labile iron pool (LIP). On the other hand, heme oxygenases (Hos) localized mainly in iron-recycling macrophages of liver and spleen, degrade heme to recover Fe^{2+}[2,13].

The regulation of hepcidin is critical in iron metabolism as binding of hepcidin to ferroportin in hepatocytes, macrophages, or enterocytes leads to internalization and degradation of ferroportin, thus limiting iron export to the blood[2,3,10]. A decrease in hepcidin when iron is needed leads to enhancement of ferroportin expression and increased iron absorption from the duodenum. In iron overload, ferroportin is downregulated and iron absorption is decreased[14]. In addition to iron deficiency, inflammatory molecules like interleukin (IL)-6 also upregulate hepcidin expression[15]. The *HAMP* gene encodes hepcidin, and its promoter is activated by the complex of bone morphogenic proteins (BMP2, BMP4, BMP6) and their receptor. This complex phosphorylates the SMAD pathway, which in turn activates *HAMP* expression[16,17]. Hemojuvelin (HJV) is a necessary co-factor for BMP-BMP receptor complex function[18]. BMP6 is mainly expressed in liver sinusoidal cells and induces hepcidin upregulation *via* paracrine signaling during iron overload[19-21].

The second receptor of transferrin (TFR2), a low-affinity receptor found in hepatocytes and erythroid precursors, is also an important inducer of hepcidin through the BMP/SMAD pathway[22-24] after forming a complex with HFE (the protein involved in hereditary hemochromatosis)[25]. Anomalies of either of the genes encoding these proteins will lead to hepcidin downregulation[26-28].

HEPCIDIN INHIBITORS

In contrast to iron overload, hypoxia, anemia, and erythropoiesis reduce hepcidin expression[29,30]. The main inhibitor of hepcidin expression is erythroferrone (ERFE)[31], which is produced by erythroid cells in response to erythropoietic stimuli. ERFE downregulates hepcidin, interfering with the BMP/SMAD pathway in hepatocytes[32-34]. Three other hepcidin inhibitors have been described. PIEZO1 and the immunophilin FKBP reduce *HAMP* expression by inhibiting the BMP/SMAD pathway[35,36]. The third hepcidin inhibitor is the ferritinophagy axis operating in both the enterocyte and the macrophage. Ferritinophagy is a specialized form of autophagy resulting in the lysosomal breakdown of ferritin and subsequent iron release to increase the LIP. It is controlled by the nuclear receptor coactivator 4

(NCOA4)[37,38] during transport of absorbed iron to ferritin. In increased iron demand, NCOA4 functions as a cargo receptor for lysosomal degradation of ferritin. Excess iron leads to lipid peroxidation-mediated ferroptosis[38]. NCOA4 is similarly involved in macrophage ferritinophagy and iron release for erythropoiesis[39].

Iron ions are dangerous to cells. In iron overload, redox-active iron increases and oxidative stress is induced through the formation of reactive oxygen species (ROS). Non-transferrin bound iron is mainly responsible for the redox-active iron when the capacity of iron binding proteins is not able to accommodate for the increased iron load. An additional dangerous form is the transit iron pool, which comprises iron that is not bound to ferritin or other chelating proteins. This iron may also induce the formation of ROS[40]. Iron is a double-edged sword[41], which even under normal conditions may cause pathological damage. Iron induces hydroxyl radical production through the Fenton reaction[42]. The Fenton-Haber-Weiss reaction is caused by the free donation and acceptance of electrons during the transition between Fe^{2+} and Fe^{3+} states. Iron-catalyzed generation of hydroxide ions and hydroperoxyl and hydroxyl radicals is the result of this exchange. Under normal conditions, free-radicals are quenched by cellular antioxidant mechanisms[43]. However, when overproduced, these free radicals promote the formation of other ROS such as thiyl and peroxyl radicals and a vicious circle is initiated leading to oxidation of lipids, proteins and nucleic acids[44]. Thus, in iron-loaded animals the products of lipid peroxidation such as malondialdehyde (MDA), isoprostanes, and 4-hydroxynonenal (4-HNE) can be detected in the liver[45]. MDA and 4-HNE form mutagenic adducts, reacting with amino groups and DNA bases[46,47] that target the p53 tumor suppressor gene initiating apoptotic resistance to the cells[48]. Levels of 4-HNE correlate well with hepatic iron levels[49]. Iron metabolism was recently reviewed in detail[50-53].

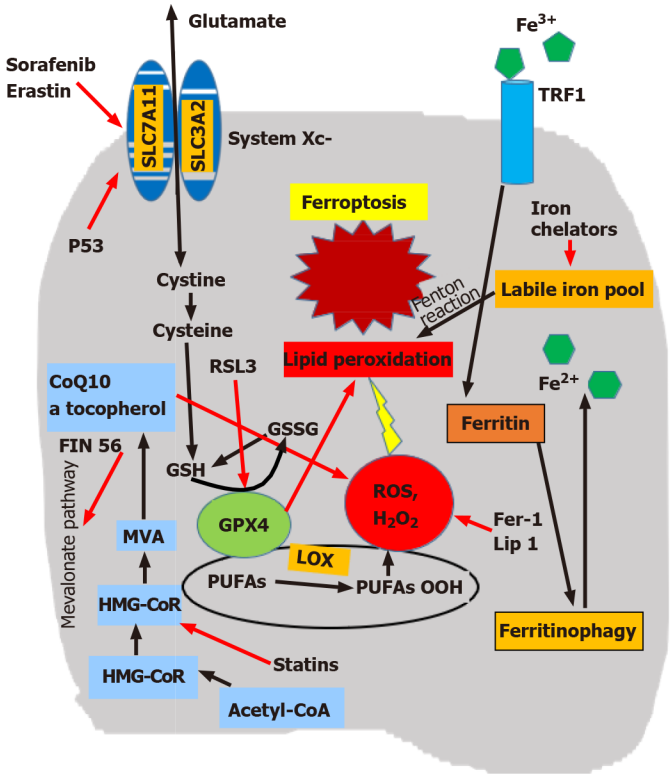
FERROPTOSIS

The most important mechanism of iron-induced liver damage is the recently described ferroptosis, a name derived from the Greek word "ptosis," meaning a fall, and the Latin "ferrum" or iron[54]. It is an iron-dependent regulated cell death characterized by iron accumulation, lipid peroxidation, and the production of ROS that depends on the activity of NADPH oxidases[55,56]. The mitochondrial respiratory chain initiates lipid peroxidation by lipoxygenase (LOX) or cytochrome P450 reductase. The enzyme glutathione peroxidase 4 (GPX4), the antioxidant glutathione (GSH), the coenzyme Q10 (CoQ10), and the tetrahydrobiopterin (BH4) system are the defense mechanisms of the cell. They are further regulated by the nuclear factor erythroid 2-related factor (Nrf2)[57-59]. The process is controlled by multiple genes associated with iron uptake[60,61], lipotoxicity[62,63], and antioxidation responses [64,65].

Ferroptosis is regulated by several metabolic events such as lipogenesis and ferritinophagy. The mitochondrial tricarboxylic acid cycle fueled by glutaminolysis may promote ferroptosis induction. Phospholipid peroxidation is the critical event in ferroptosis. Production of ROS, iron, and phospholipids containing polyunsaturated fatty acids (PUFA-PLs) are the necessary requirements. The executioner of ferroptosis is phospholipid hydroperoxide (PLOOH) synthesized from its precursor, PUFA[66].

Both non-enzymatic/exogenous and enzymatic/endogenous pathways are implicated in lipid peroxidation. For the latter, LOXs and/or cytochrome P450 oxidoreductase mediate the induction of lipid peroxidation by the dioxygenation of lipids. Exogenous transporter mediated signaling pathways include the E cadherin-NF2-Hippo-YAP pathway, the glucose-regulated AMPK signaling pathway, and the p53 tumor suppressor pathway[67].

Mechanisms inhibiting ferroptosis are provided by three main biological pathways (Figure 1)[68,69]. The first is the GSH/GPX4 pathway, implicating the system Xc-, which is a membrane cystine/glutamate exchanger that imports cystine and exports glutamate. A critical mediator in this system is the cystine/glutamate antiporter SLC7A11, and GPX4 is the major protective system against lipid peroxidation[70]. In addition, ferroptosis suppressor protein 1 acts mainly on the plasma membrane, and dihydroorotate dehydrogenase is an important defense molecule in mitochondria[71-75]. The second comprises iron metabolism pathways, particularly the p62-Kelch-like ECH-associated protein 1 (Keap1)-Nrf2 regulatory pathway[54]. Inhibition of ferritinophagy increases mitochondrial ferritin and protects from ferroptosis as evidenced in hypoxic macrophages. This is regulated by a hypoxia-induced decrease of *NCOA4* transcription, in combination with a microRNA 6862-5p-dependent degradation of NCOA4 mRNA[76]. Nrf2 is a transcription factor that protects cells against oxidative and toxic damage and plays a significant role in regulating ferroptosis[77-79]. In hepatocellular carcinoma (HCC) and other tumors, activation of the p62-Keap1-Nrf2 pathway leads to reduced Nrf2 degradation, the protection of tumor cells against ferroptosis, and resistance to anticancer drugs[80]. The third includes lipid metabolism pathways implicating p53 and various enzymes[54,66]. p53 is a tumor suppressor transcription factor that may prevent cancer by controlling the cell cycle, cellular senescence, and apoptosis. Ferroptosis is one of its antitumor mechanisms; p53 increases cell sensitivity to ferroptosis through repression of *SLC7A11*. The ferroptosis inhibitor fer-1 reverses this effect and induces *SLC7A11*



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Figure 1 Simplified pathways of ferroptosis and site of action of the main ferroptosis modulators. Ferroptosis is mediated through excess intracellular iron in the labile iron pool (LIP). Ferritinophagy is a major source of LIP, leading to the production of reactive oxygen species (ROS) by mitochondria and lipid peroxidation. Deranged function of the system Xc- causes reduction of glutathione (GSH) and glutathione peroxidase 4 (GPX4) and an increased activity of lipoxygenases (LOXs), augmenting lipid peroxidation. Ferroptosis modulators act by interfering with components of this complicated pathway. Red arrows indicate inhibition. CoQ10: Coenzyme Q10; GSSG: Glutathione disulfide; MVA: Modified vaccinia virus Ankara; PUFAs: Polyunsaturated fatty acids; SLC3A2: Solute carrier family 3 member 2; SLC7A11: Solute carrier family 7 member 11; TRF1: Transferrin receptor 1.

overexpression[62,81-83]. Additional biological factors inhibiting ferroptosis were also recently identified: (1) GTP Cyclohydrolase-1 (the rate-limiting enzyme for biosynthesis of tetrahydrobiopterin (BH4), which counteracts ferroptosis[84]); (2) Transferrin and its cell surface TFR1 receptor[12]; and (3) CDGSH (iron sulfur domain 1, which negatively regulates ferroptosis protecting against lipid peroxidation in mitochondria)[85].

EXOGENOUS FERROPTOSIS MODULATORS

Exogenous ferroptosis modulators discussed here are summarized in Table 1. Ferroptosis inhibitors are divided into two major groups: (1) Class I inhibitors, such as Deferoxamine (DFO) mesylate[86], which suppresses iron accumulation; and (2) Class II inhibitors, including ferrostatin-1, liproxstatin-1 and vitamin E, which react with chain free radicals and can inhibit lipid peroxidation[87-91]. The activity of drugs in the first generation of ferrostatin class specifically reduce the accumulation of lipid ROS. The second generation (SRS 11-92) and the third generation (SRS 16-86) ferrostatin drugs function through conferring increased metabolic stability[90].

The recently described inhibitor dynasore has characteristics of both classes, and prevents both iron accumulation and lipid peroxidation[92]. Other inhibitors of ferroptosis have been identified. For example, the cholesterol-reducing drug probucol was found to suppress ferroptosis[93]. The RIPK1 inhibitor necrostatin-1, which suppresses necroptosis, also has the additional effect of suppressing ferroptosis. Selenium administration has also been seen to suppress ferroptosis during stroke[94], and the nitroxide XJB-5-131 targets mitochondria and suppresses both apoptosis and ferroptosis[95]. However, it should be noted that these inhibitors have not been tested in the liver.

Interestingly, a recent experimental finding showed that the mechanism of action of bicyclol, a common hepatoprotectant in China, is *via* the prevention of ferroptosis. Furthermore, bicyclol attenuates cellular damage and lipid peroxidation induced by erastin. Additionally, Nrf2 inhibition and the subsequent reduction of GPX4 levels impedes the effects of bicyclol[96]. Finally, the anti-diabetic drug rosiglitazone inhibits ferroptosis and reduces hepatocyte death, acting as an ACSL4 inhibitor[97,98].

Table 1 Exogenous modulators of ferroptosis

Inducers	Mechanisms	Compounds
Class 1	Inhibition of system Xc-	Erastin, sorafenib, sulfasalazin
	Prevention of cystine import	Glutamate
Class 2	Inhibition of GPX4	RLS3, DPIs (DPI7, DPI10)
Class 3	Degradation of GPX4	FIN56
	Depletion of CoQ10	
Class 4	Initiation of lipid peroxidation	FINO2, PUFAs
	Indirect reduction of GPX4 activity	
Inhibitors		
Class 1	Suppression of iron accumulation	Deferoxamine
Class 2	Inhibition of lipid peroxidation	Ferrostatin-1, liproxstatin-1, vitamin E
Unclassified		Dynasore, probucol, selenium, nitroxide XJB-5-131
		Bicyclol, rosiglitazone

CoQ10: Coenzyme Q10; FIN: Ferroptosis inducing compounds; GPX4: Glutathione peroxidase 4; PUFAs: Polyunsaturated fatty acids.

Ferroptosis inducers

Class I inducers such as erastin, sorafenib, sulfasalazine and glutamate, deplete cellular cysteine by inhibiting system Xc- and the biosynthesis of GSH, resulting in the loss of GPX4 activity[81,99-102]. The low water solubility and metabolic instability of erastin has limited its clinical application[103], but a metabolically stable erastin derivative has been tested[104].

Class II inducers, including RSL3 and DPI compounds, act by directly inhibiting GPX4[88,105-107], leading to the accumulation of lipid peroxides and eventual cell death. BSO and cisplatin also deplete GSH inducing ferroptosis. Cisplatin and erastin have a significant synergistic effect[108]. Interestingly, erastin promotes ferritinophagy and increased the free iron, lipid peroxidation, while RSL3 does not interfere with ferritinophagy, suggesting that RSL3 induction of ferroptosis is not dependent on ferritin degradation[109].

Class III inducers such as FIN56 act by both direct degradation of GPX4 and indirect inactivation of GPX4 *via* the squalene synthase-mevalonate pathway of the mitochondrial electron transport chain[103,110]. FIN56 also acts by depleting GPX4 and CoQ10. It seems that the cellular lethality of FIN56 is increased when cells are co-treated with statins and FIN56[110]. In addition, statins, such as simvastatin, enhance ferroptosis by inhibiting HMG-CoA reductase[110].

In class IV inducers, ferroptosis is induced by excess iron, omega-3 PUFAs, or peroxides, such as FINO2, that initiate lipid peroxidation and indirectly reduce GPX4 activity[111,112]. FINO2 is the only class IV ferroptosis inducer tested so far, but several other have been synthesized[103]. PUFAs show anticancer activity[113], but shortcomings such as reduced bioavailability, limited resistance to oxidative degradation, and lack of uptake specificity impede their use. However, the application of nanotechnology improves their therapeutic use[114]. Low density lipoproteins (LDLs) are taken up by LDL receptor expressed in tumor cells. LDL-based nanoparticles with docosahexaenoic acid (LDL-DHA NPs) were found to maintain their stability and specificity[115,116].

Experimental evidence suggests that there are additional biological inducers of ferroptosis, but their significance in human disease is still unknown. As mentioned above, ferritinophagy is a special recycling process of autophagy for the autophagic degradation of ferritin in lysosomes. It is mediated by the autophagic cargo receptor NCOA4, and leads to the initiation of ferroptosis[117]. Augmented ferritinophagy mediated by an increase of NCOA4 leads to induction of ferroptosis[64] (Table 1).

Reduction of iron-response element binding protein 2 significantly reduces erastin induced ferroptosis[55]. Increased activity of HO-1, the enzyme responsible for degradation of heme into ferrous iron, carbon monoxide, and biliverdin, increases LIP and initiated ferroptosis[118,119]. Artesunate (a derivative of artemisinin) is used in severe malaria[120] and induces hematopoietic stem cell (HSC) ferroptosis. However, the malaria drug chloroquine (a ferritinophagy inhibitor) reverses this effect, implying that artesunate induces HSC ferroptosis by activating ferritinophagy[121].

Finally, magnesium isoglycyrrhizinate (MgIG) is a natural product with anticancer activity[122] that has been shown to promote HSC ferroptosis. Inhibition of HO-1 reduces MgIG-induced HSC ferroptosis, suggesting that the promotion of HSC ferroptosis is mediated through upregulation of this enzyme[123].

LIVER MACROPHAGES IN IRON METABOLISM AND FERROPTOSIS

Kupffer cells and other liver and spleen macrophages take up heme from damaged or senescent erythrocytes and either export the extracted Fe^{2+} using ferroportin or store it in ferritin in the cytoplasm [124]. It has been shown that intracellular iron regulates the differentiation of macrophages into M1 (pro-inflammatory) and M2 (anti-inflammatory) subtypes[125,126]. M1 macrophages have an iron storage capability with higher HAMP but lower FPN and IRP1/2 compared to M2 subtype[127]. M1 polarization is regulated by iron overload[128], but also by ROS production and p53 acetylation induced by iron overload[129]. Recently, experiments with cultured macrophages demonstrated that chronic iron overload may in fact downregulate M1 markers and show signs of M2 differentiation[130].

During infection, hepcidin blocks macrophage differentiation to reduce iron export that could increase the growth of pathogens[131], which is reversed in the case of intracellular pathogens. This is possibly achieved by an increased production of nitric oxide[132] and the expression of the phagolysosomal protein NRAMP1 both leading to induction of ferroportin and intracellular iron reduction [133].

Kupffer cells exhibit phagocytic dysfunction and impair iron homeostasis during the development of non-alcoholic fatty liver disease (NAFLD)[134-136]. In addition, they participate in the clearance of lipids in nonalcoholic steatohepatitis (NASH) through M1 differentiation with the help of invariant natural killer T cells[137-139]. This composite role indicates that Kupffer cells can influence the development of ferroptosis, providing a new target for therapy in NAFLD.

Moreover, acute iron deprivation led to changes in metabolic and immunoregulatory genes in human macrophages resulting in impaired cell proliferation and reduced inflammation[140]. This is in contrast to the pro-inflammatory production of leukotrienes by the enzyme 5-LOX mediated by ferric iron in human macrophages[141]. As expected, ferroptosis has been the subject of several detailed reviews[69, 142-145], which include descriptions of ferroptosis regulators[146,147], ferroptosis in viral disease[148], and the role of macrophages in ferroptosis[149].

IRON IN LIVER DISEASE

Patients with chronic liver disease may exhibit hepatic and splenic iron loading, usually inside Kupffer cells and splenic and bone marrow macrophages[150]. Sometimes, this is accompanied by low hemoglobin levels and other hemolysis indices, indicating that hemolysis may have a role in the development of secondary iron overload[151]. However, a recent review emphasized the role of low levels of hepcidin in various liver diseases as implicated in both iron deposition in hepatocytes and participation in stellate cell activation and liver fibrosis[152].

Excess free iron exerts a toxic effect on the liver, favoring the progression of liver disease[58,153], and indeed abnormalities of iron regulation are reported in various liver diseases apart from inherited hemochromatosis[154]. Hyperferritinemia has been the main manifestation of disturbed iron homeostasis in chronic liver disease[155,156].

Opposing views have also been expressed in the literature. Data from cell culture experiments and animal models suggest that iron overload is only a weak fibrosis inducer and rarely causes serious liver damage not supporting the concept that iron overload is an important cause of liver toxicity. Iron may co-exist with other causes of inflammation, and the resulting hepatocyte necrosis is the real driving force leading to fibrosis[157].

The role of iron overload and the significance of ferroptosis have been investigated in the context of several liver diseases. The most common liver diseases will be discussed as well as the common end points of all, namely cirrhosis sometimes followed by the development of HCC. The rather limited available information on other liver diseases will be presented.

NAFLD/NASH

The role of iron in liver damage has been extensively researched in the case of NAFLD. A new term was introduced, the dysmetabolic or insulin-resistance hepatic iron overload syndrome (DIOS or IR-HIO), which is characterized by high serum ferritin levels, unexplained iron overload, and is associated with metabolic abnormalities[158-161]. IR-HIO is detected in one third to half of patients with NAFLD[155, 158,162,163]. The reason for the observed iron overload in NAFLD is still uncertain. A proposed mechanism is the redistribution of transferrin receptors (TfRs) to the cell surface, a process induced by insulin[158,163,164]. Tfr1 is upregulated in mice on a high fat diet, which may enhance hepatocellular iron uptake in NAFLD despite already increased hepatocellular iron[165]. The increase in serum ferritin may be due to increased iron stores, oxidative stress caused by lipid abnormalities, systemic inflammation, and genetics[166,167]. The implication of the presence of the Cys282Tyr *HFE* gene variant of hereditary hemochromatosis was also examined. A heterozygous mutation is associated with bridging fibrosis or cirrhosis in Caucasians[168-170]. By contrast, in knock out mouse models of hemochro-

matosis no progression to steatohepatitis or liver fibrosis was noted with a high-fat diet[171].

In addition, certain variants of ceruloplasmin are associated with increased liver iron stores and high ferritin in patients with NAFLD and advanced liver fibrosis[172,173]. Ceruloplasmin mutations have been associated with iron deposition in the liver of other chronic liver diseases as well[174]. Excess dietary iron causes hepatic oxidative stress, inflammation and hepatocellular ballooning injury leading to NASH[175,176]. Oxidative stress interferes with mitochondrial function, impairing fatty acid oxidation and producing different pro-inflammatory factors such as tumor necrosis factor (TNF)-α, IL-6, IL-8, MDA and nitric oxide[177-180], leading to NASH. Moreover, liver iron deposition increases cholesterol synthesis, lipid accumulation, and impairs cellular stress responses, which further exacerbate NAFLD[181-184].

The pattern of hepatic iron deposition is important in NAFLD patients, as iron deposition in macrophages is associated with more advanced disease[185]. An important observation was recently reported emphasizing the role of liver macrophages in the pathogenesis of NASH. A histological structure, the crown-like structure, has been described in NASH: Iron-rich Kupffer cells surround dead hepatocytes, take up debris, and induce inflammation and fibrosis. They have proinflammatory and profibrotic phenotypes, driving liver fibrosis[186]. Hepatic iron was significantly higher in patients with HCC associated with NASH and it was mostly localized in Kupffer cells[187]. Evidence suggests that iron may contribute to NAFLD pathogenesis and fuel the progression to NASH[178-180].

Red blood cell fragility and erythrophagocytosis may also explain iron deposition in NAFLD. It could be the result of insulin resistance and membrane lipid abnormalities[188]. Recently, aristolochic acid-associated drugs (atypical antipsychotic medications) were reported to induce NAFLD and link insulin resistance with iron metabolism dysregulation irrespective of drug-associated weight gain[189]. Regardless, whatever the etiology of the iron deposition in NAFLD and NASH, the clinical consequences are well documented.

Hyperferritinemia is also frequent in patients with NAFLD. Sometimes, it is the first laboratory abnormality leading to further clinical investigation[190]. In a large prospective population-based study from South Korea, serum ferritin was a strong early predictor of future development of steatosis, indicating that the ferritin association with NAFLD is not a simple consequence of the disease itself [191]. Patients with high ferritin have more severe steatosis[192,193], inflammation[194], advanced fibrosis[195], and increased mortality[196,197]. It has been suggested that serum ferritin could be used as a marker to identify NAFLD patients likely to have NASH and fibrosis[166]. However, a clear association between serum ferritin and fibrosis could not be verified in other studies that reported that ferritin could not accurately predict advanced fibrosis in NAFLD[198,199,200]. This discrepancy may be explained by the findings of a recent investigation in which hyperferritinemia was found in a quarter of NAFLD patients. In this study, stainable iron was present in hepatocytes, Kupffer cells, or more frequently, in both. Importantly, serum ferritin was not related to the presence of NASH, but it increased with worsening of fibrosis and decreased in the cirrhotic stage of the disease[201].

Iron measurement by magnetic resonance imaging (MRI) demonstrates that liver iron is the most important determinant of serum ferritin in NAFLD[202]. An important association of serum ferritin with the gut microbiome was also recently reported. In this study, ferritin levels were associated with differences in gut microbial composition. Both negative and positive associations with particular microbial species were found, and ferritin-related bacterial species correlated with hepatic iron-related genes. Moreover, the iron-associated microbiome was also linked to liver fat load. Fecal transplantation from high-ferritin mice to normal mice confirmed the human results and demonstrated an interplay among iron load, liver fat, and gut microbiome that could be exploited in future treatments[203].

Hepcidin in NAFLD

As in other liver diseases, extensive research has been conducted on the possible role of hepcidin in NAFLD. Investigations have tried to identify if the reported hepcidin abnormalities were the cause or the result of the iron overload observed in many cases of NAFLD. In various studies, hepcidin has been demonstrated to be either increased or decreased in NAFLD. In obese individuals, adipose tissue expression of hepcidin was upregulated, irrespective of steatosis and NASH. The contribution of adipose tissue hepcidin to the serum hepcidin is not well studied, but it may potentially explain the increased serum hepcidin in NAFLD[182,204-207].

Furthermore, leptin was found to correlate with hepcidin levels in obese children. Leptin also upregulates hepcidin expression in hepatocyte cultures, indicating that an increase in hepcidin may correlate to the leptin abnormalities in NAFLD[208,209]. Hepcidin downregulation, on the other hand, may be a consequence of oxidative stress secondary to iron overload[158-160,208,210]. Experimental evidence has demonstrated that hepcidin downregulation is a secondary phenomenon occurring after deposition of iron in the liver and the concomitant increase in oxidative stress[211]. Furthermore, an investigation on the relationship between iron stores and cardiovascular damage in patients with NAFLD showed that ferritin was associated with the components of the metabolic syndrome but not with liver inflammation and damage. In this study, hepcidin was increased due to the increased iron load[198], and fat in the liver of mice increased the expression of BMP-binding endothelial regulator, which was produced in sinusoidal endothelial cells and inhibited the BMP-SMAD pathway leading to a secondary inhibition of hepcidin. This is an additional explanation for the iron deposition in NAFLD

[212].

Clinical data also indicate that hepcidin abnormalities are not the primary cause of the excess iron in the liver observed in NAFLD. HJV levels were low and hepcidin levels were high in iron-overloaded NAFLD patients. These findings support the suggestion that iron accumulation may be the primary inciting event in this disease[213].

Individuals with the metabolic syndrome preserve the iron regulatory control of hepcidin, and hepcidin progressively increases in response to the increase of iron stores[205,214-216]. In addition, serum hepcidin and HAMP mRNA in the liver correlate to body iron stores irrespective of the degree of iron deposition. Thus, the dysmetabolic iron overload syndrome (DIOS) syndrome seen in NAFLD is not related to altered hepcidin synthesis[217].

However, despite the elevated serum hepcidin, duodenal iron absorption is increased because DMT1 is upregulated by IRP1 activation, likely due to unidentified humoral factors in the sera of NASH patients[218]. It seems, therefore, that elevated hepcidin in NAFLD is either a reflection of hepatocellular inflammation in NASH, or that increased iron and the associated induction of hepcidin appears before the development of NAFLD or NASH[219].

So far, data suggest that the interplay between iron and lipid metabolism is multifaceted in NAFLD. Moreover, it could be suggested that iron is directly implicated in NAFLD pathogenesis. Reports that increased dietary iron from red meat may predispose individuals to type II diabetes and insulin resistance are supportive evidence for such an idea[220-222].

Contrasting results have also been reported. For example, inadequate hepcidin production in response to a given level of iron load in NAFLD patients compared to controls was found in one study [159]. An impairment in the ability of hepcidin to inhibit iron absorption was also demonstrated in DIOS, suggesting hepcidin resistance in this condition[223]. The recent description of ferroptosis has prompted new investigations on the effects of liver iron load in NAFLD, although its exact role in this disease process has not been fully clarified.

Ferroptosis was recently related to the induction of inflammation in the early stages of NASH, making it a possible the “first hit” in its pathogenesis[98]. Further studies indicated that ferroptosis plays a critical role in the progression of NASH, making it a promising treatment target[224,225]. The enzyme arachidonate 12-LOX is known to promote the progression of NASH[226,227], and arachidonic acid metabolism has been shown to trigger ferroptosis in a diet-induced NASH mouse model[224]. Furthermore, the levels of the central regulator of ferroptosis ACSL4 were increased in a rat NASH model, and inhibition of the Mfn2/IRE1αACSL4 pathway was found to prevent incidence and development of NASH[228]. However, the connection between NAFLD and ferroptosis is still debatable, and many reviews of iron and NAFLD pathogenesis have been presented[229-231].

ALCOHOLIC LIVER DISEASE

Early reports showed that stainable iron is present in the livers of alcoholics[232,233]. Hepatocyte iron deposition is considered an important feature of alcoholic liver disease (ALD), although stainable iron in Kupffer cells is more prominent, particularly in the advanced stages of disease[234]. Ethanol consumption triggers iron overload[235]; it has been shown in patients with ALD that ethanol increases iron uptake from circulating de-sialylated transferrin by hepatocytes[236].

Almost half of patients with ALD have hepatic iron overload (HIO)[237] with high values of plasma ferritin and transferrin saturation[238,239]. Drinkers, from an early age, have increased iron markers [208,240]. High liver iron was found to be predictive of HCC development or death in patients with alcoholic cirrhosis[241,242], often acting synergistically with diabetes mellitus and viral hepatitis[243, 244]. Ethanol is metabolized into acetaldehyde, forming DNA and protein adducts that predispose individuals to HCC[103]. Iron is directly implicated in HCC development, since it accumulates in lysosomes through ferritinophagy and reaches the cytoplasm as free iron[245]. The resultant production of free radicals through the Fenton reaction initially activates Kupffer and stellate cells, ultimately leading to ferroptosis[246]. Additional significant production of ROS is mediated by cytochrome P450 2E1 (CYP2E1), which is directly induced by alcohol[247]. Alcohol consumption results in up to 20 folds increase of CYP2E1[248]. Additional mechanisms of alcohol-induced HCC have also been reviewed [249].

The question of increased iron load in the liver of patients with ALD has prompted research on hepcidin regulation in ALD. Suppression of hepcidin expression by ethanol has been reported in cell culture and experimental animal models, possibly *via* the inhibition of CCAAT enhancer binding protein-α (C/EBP-α)[250-253]. Iron induces activation of C/EBP-α, but ethanol inhibits this action and leads to inadequate hepcidin expression[254]. Suppression of the BMP6/SMAD pathway by alcohol has also been reported[255]. Hepcidin downregulation is also mediated by the induction of oxidative stress caused by either the effects of ethanol itself or free iron. As such, antioxidant treatment attenuates hepcidin downregulation[citation]. Ethanol may also increase hepatocyte iron uptake by upregulating the expression of TfR[246], even in habitual drinkers[256]. Additionally, ethanol may reduce hepcidin through proteins involved in liver regeneration; however, this requires further investigation[257].

Ethanol exposure simultaneously increases the expression of DMT1 and FPN in the duodenum[254], which has been linked to liver fibrosis[254,258]. Iron absorption is increased two-fold in chronic alcoholics[259], and ethanol administration in a mouse model overexpressing adipose tissue lipin-1 accelerated iron accumulation followed by lipid peroxidation, reduction of GSH, and induction of ferroptotic liver damage[260].

The effect of ethanol on hepcidin seems to be more complex than previously thought[261]. Ethanol has been shown to increase transforming growth factor (TGF)-β expression and phosphorylation of SMAD2[262]. Increased activation of SMAD2/3 can abrogate the TGF-β-induced hepcidin upregulation [263]. Hepcidin is also suppressed by ethanol through the toll-like receptor 4 (TLR4) pathway, and ethanol does not suppress hepcidin in TLR4 receptor mutant mice[264]. Interestingly, TLR4 deficiency has been shown to protect animals from liver fibrosis[265,266]. Further evidence suggests that ethanol action on TLR4 involves HSCs, as TLR4 on Kupffer cells or mature hepatocytes are unlikely targets of the effects of ethanol[267,268].

Both serum transferrin and serum hepcidin have been used as prognostic markers in ALD. To this end, low transferrin levels[269,270] have been associated with worse prognosis[197,270-272]. Importantly, the prognostic value of serum transferrin is similar to other traditional prognostic scores, such as the model of end-stage liver disease (MELD) and the Glasgow alcoholic hepatitis scores[273].

The recent identification of ferroptosis has allowed for a better understanding of the connection between lipid and iron abnormalities observed in ALD[274]. Ferroptosis is downregulated during the repair of ethanol-induced liver damage, while ferroptosis inhibition or activation of the Nfr2 pathway reversed ROS accumulation and lipid peroxidation induced by ethanol[275,276]. Excessive ethanol activates genes like frataxin that promote liver injury[277]. More importantly, ferroptosis provides a strong link for the recently demonstrated crosstalk between the liver and the gut[278]. Lack of intestinal sirtuin 1 has been shown to limit ferroptosis, normalize iron overload, and ameliorate ethanol-induced liver damage[279]. Ferroptosis is also implicated in adipose-liver axis abnormalities observed in alcoholic steatohepatitis[260]. Moreover, the overexpression of adipose-specific lipin-1 aggravates alcoholic steatohepatitis and iron deposition, increasing hepatic MDA levels[153,260].

Finally, an additional mechanism of ethanol-induced liver damage has been identified in severe alcoholic hepatitis patients. Iron overload triggers activation of the metallopeptidase ADAM17, which leads to the increase of TNF-α and soluble CD163, resulting in macrophage activation and promotion of hepatic inflammation[280]. Detailed reviews on iron and ALD were recently published[143,281,282].

CHRONIC HEPATITIS C

The effect of iron on the activity and infection cycle of the hepatitis C virus (HCV) has been controversial. Inhibition of viral replication by iron due to the suppression of the nonstructural protein 5B has been reported[283], but enhancement of viral replication has also been observed[284]. HCV alters the expression of hepcidin and therefore cellular iron metabolism[285,286]. Experimental evidence in early HCV infection has demonstrated increased hepcidin expression followed by enhanced viral translation and replication. In this study, iron loading of macrophages accompanied hepcidin upregulation and resulted in increased viral transmission to naïve cells[287]. Other experimental studies, however, have shown that hepcidin levels are low in HCV-infected cell lines[288,289].

Inhibition of hepcidin expression has been attributed to HCV-induced oxidative stress[290,291]. Experiments in chimpanzees on high iron diets have demonstrated that liver damage is observed only in animals infected with HCV, indicating a harmful effect of iron in HCV infection[292]. In chronic infection, HCV interferes with the expression of the iron uptake receptor TfR1, a known mediator of HCV internalization[293,294]. The observed downregulation of hepcidin despite hepatic inflammation in chronic HCV[295] may be related to impairment of the BMP6/HJV pathway by TNF-α, which would suppress the transcription of HJV[296].

Clinical studies have verified that HCV infection downregulates hepcidin[297-299], and serum hepcidin has been correlated with severity of liver disease[300]. More than 40% of patients have iron overload associated with a high rate of liver damage and inflammatory activity, as well as an increased risk of hepatocarcinogenesis[301-303]. Hepatic iron and HCV proteins in combination produce a toxic hydroxyl radical ($^{\cdot}$OH) that forms mutagenic bases such as 8-hydroxy-2-deoxyguanosine (8-oxodG)[304, 305]. HCV patients have been shown to have an approximately 10-fold increase of 8-oxodG in liver tissue compared to non-HCV control patients[306].

The Hepatitis C Antiviral Long-Term Treatment Against Cirrhosis trial has convincingly demonstrated that iron in hepatocytes and portal tract cells predicts progression to decompensated cirrhosis, HCC, and death[307]. Almost all liver tissue from HCV patients had some lysosomal iron deposits detected by electron microscopy and X-ray microanalysis, despite negative results with classical Prussian Blue staining[308]. Moreover, increased serum aminotransferases were found only in HCV patients with stainable iron in Kupffer cells but not in those with hepatocellular iron[309].

Even minor increases in iron load in heterozygous carriers of C282Y or H63D gene mutations for hemochromatosis were found to induce more fibrosis in chronic HCV infection[310,311]. Genotype 3-

infected patients have more frequently elevated liver iron, which has been associated with hepatic steatosis in this type of HCV infection[312]. Evidence from thalassemia patients further indicates that iron adversely affects the disease course of HCV, increasing morbidity and mortality due to more severe liver disease[313].

Liver iron also adversely affects the response to interferon (IFN)-based treatments[314]. In studies involving IFN treatment, ferritin levels increased regardless of sustained virologic response (SVR) and decreased at about 3 years post-treatment. This is not the case with direct-acting antivirals (DAAs)[315,316], where SVR is achieved irrespective of iron status[317-320]. A recent study demonstrated that pre-treatment elevated serum ferritin and ERFE levels were restored after treatment with DAAs and correlated with changes in LDL cholesterol levels, but only in men[321].

Plasma ferritin, liver iron, and transferrin saturation are also increased in HCV infection[322] and elevated serum ferritin has been related to liver fibrosis[323]. An additional reason for increased liver iron in HCV patients is the reported increased hemolysis particularly in advanced stages of the disease [151]. Despite the evidence presented above, different results in relation to the role of HCV-induced iron overload have been presented[324,325]. In several studies, elevated serum ferritin and hepatic iron played no significant role in the progression of liver damage[326,327]. Moreover, the significance of hemochromatosis mutations has been questioned as a risk factor in the progression of HCV-related disease[328].

Recently, it has been suggested that ferroptosis may be implicated in the natural course of HCV[58]. Importantly, HCV replication is inhibited by an iron-dependent mechanism like ferroptosis, which is mediated by the desaturation of oleate to highly unsaturated fatty acids by the enzyme fatty acid desaturase 2 (FADS2). This is a key determinant of cellular sensitivity to ferroptosis; FADS2 suppression significantly enhances HCV replication, whereas the ferroptosis inducer erastin sensitizes HCV to DAAs, altering the conformation of HCV replicase[329].

CHRONIC HEPATITIS B

Iron favors hepatitis B virus (HBV) mRNA expression in HepG2 cells[330]. Increased serum and cellular iron uptake and decreased hepcidin expression have been reported in HBV infection[297,331]. Hepatitis B-infected patients frequently show iron deposition in hepatocytes and elevated liver iron concentration (LIC) leading to increased disease severity[332,333]. Serum ferritin levels are also increased in patients with chronic HBV[332].

Levels of hepcidin are increased in early stages of HBV and reduced in the cirrhotic stage[334,335]. Co-infection with hepatitis D increases the iron load[332]. However, results of studies regarding serum hepcidin in HBV infection are not uniform. Reduced serum hepcidin has been reported in HBV patients with or without cirrhosis[336], while another report found that hepcidin is slightly increased in HBV patients without cirrhosis and in those with HCC[335]. The reason for this discrepancy is not clear. Nonetheless, decreased hepcidin levels and elevated transferrin saturation and ferritin levels have been associated with fibrosis severity in patients with chronic HBV[337].

It should be noted that iron deposition in the liver has been considered a secondary phenomenon. Damaged hepatocytes in viral hepatitis undergo necrosis and the released iron is scavenged by Kupffer cells[240,338]. However, this mechanism cannot entirely account for the deposition of iron in hepatocytes. The implication of HBV in iron deposition is exemplified in a case report in which a female patient with symptoms of iron overload had highly increased serum ferritin and transferrin saturation. In this case, all of the patient's symptoms resolved and her iron abnormalities normalized after HBV antiviral monotherapy[339].

LIVER FIBROSIS AND CIRRHOSIS

Nearly 6 decades ago, it was shown that iron on liver biopsy is associated with manifestations of advanced disease compared to that in non-iron overloaded cirrhotic patients[340]. Cirrhotic patients with hemosiderosis are more likely to be classified as Child Pugh class B or C with higher MELD scores than those without stainable iron[341,342].

As mentioned before, hyperferritinemia and high liver iron predict the risk of advanced liver fibrosis in NAFLD[166,179,343]. A recent study of a large number of NAFLD patients with a long follow-up (mean 8.4 years) emphasized the fact that it is the non-parenchymal iron deposition that leads to serious liver disease[344].

Fibrosis is increased by the presence of iron through increased HSC proliferation and selectively increased collagen synthesis without interference by non-collagen proteins[345,346]. Experiments with cultured HSCs have shown that incubation with either ferritin or transferrin increases nuclear factor kappa-B translocation and HSC activation[347,348], and enhances α-smooth muscle actin, collagen, and vimentin synthesis[349]. Isoprostanes, products of arachidonic acid peroxidation produced during iron-induced oxidative stress, increase HSC-collagen-production and TGF-β release from Kupffer cells[350].

Furthermore, 4-HNE upregulates the expression of collagen and the TIMP-1 inhibitor of metalloproteases in HSCs[351].

Elastin, another component of the extracellular matrix, is also affected by iron. Elastogenesis is modulated in cultured human skin fibroblasts by iron, as evidenced by the levels of both elastin protein and elastin mRNA are increasing 3-fold[194]. Liver iron load also induces both TGF-β[352] and BMP-6 [353,354]. The connection between fibrosis and hepcidin pathways and the significance of SMAD4 as their common link has been demonstrated[353]. Other signaling pathways related to fibrosis are also modulated by iron. For example, iron deficiency stimulates Notch signaling[355], and recently, iron-loading revealed a protective role of β-catenin (a component of the cadherin complex that stimulates Wnt signaling) against liver fibrosis[356]. Hepcidin also has a protective role in liver fibrosis by suppressing HSC activation[357]. BMP6, the main hepcidin inducer, has a similar protective role in fibrosis inhibiting HSCs activation[358]. Evidence regarding the role of ferroportin in liver fibrosis is limited. However, ferroportin has been shown to be increased in activated HSCs and the anti-fibrotic action of hepcidin in HSCs mentioned above may be mediated by degradation of ferroportin[357].

Clinical evidence confirms the importance of iron metabolism in the development of fibrosis. For example, ferritin levels have been associated with decompensation and increased mortality in cirrhosis [359]. However, ferritin concentration has poor sensitivity as a marker of liver fibrosis, since it also increases as a result of inflammation[360]. Transferrin also has clinical significance in HCV- and HBV-related cirrhosis; it has been associated with advanced fibrosis and is a predictor of survival in cirrhotic patients[269,301,338]. Additionally, low hepcidin levels can cause iron overload and increased oxidative stress in the liver[361], which in combination with other factors such as genetic variables, viral infections, and alcohol use, can eventually lead to liver fibrosis[362].

Low hepcidin has also been demonstrated as a predictor of mortality and development of HCC in alcoholic cirrhosis[258,363]. Similarly, in HBV cirrhosis, hepcidin is low compared to patients without cirrhosis[335,364], where values are similar to healthy controls[335,365]. In HCV-related cirrhosis and alcoholic cirrhosis, hepcidin is significantly lower than in HBV cirrhosis[365-367].

Hepcidin levels are not reduced in the early stages of NAFLD, but eventually drop in advanced fibrosis, similar to what has been observed in other liver diseases[368]. Unlike ferritin[369], serum hepcidin is a reliable marker of severity of fibrosis in NAFLD[368,370]. A low hepcidin/ferritin ratio can differentiate between cirrhosis and non-cirrhosis in patients with HBV, HCV and NAFLD[367], but not in ALD patients, possibly because ethanol directly inhibits hepcidin expression as mentioned above.

Ferroptosis

The role of ferroptosis in liver fibrosis was recently investigated. Its role is debatable as both induction and attenuation of liver fibrosis by ferroptosis has been reported. Ferroptosis increased susceptibility to fibrosis in mice on a high-iron diet, an effect reversed by a ferroptosis inhibitor[12]. However, other studies have shown that ferroptosis attenuates HSC activation and reduces liver fibrosis. Moreover, the ferroptosis inducers erastin and sorafenib reduce liver fibrosis increasing ferritinophagy[371], and MgIG increases ferroptosis, leading to reversion of fibrosis[123]. The anti-malarial agent artemether increases the p53-dependent ferroptosis and inhibits HSC activation[372] and artesunate, a derivative of artemisinin with immunomodulating properties, induced ferroptosis of activated HSCs possibly triggering ferritinophagy[121]. The role of iron in liver fibrosis has been recently reviewed[152,373].

HCC

Hepatic iron overload has long been linked to HCC tumorigenesis and tumor growth[147,374-376]. Iron incubation of an HCC cell line has been shown to increase mesenchymal and metastatic markers, representing a fundamental defect in cancer development[377]. Patients with hereditary hemochromatosis show a 20-200-fold increase risk of HCC development[378,379]. Additionally, iron score has been demonstrated to be significantly higher in HCC-NASH patients than in NASH controls[343]. In HCC patients, iron localization is mainly sinusoidal[187], and iron deposition in the portal tract has been associated with poor survival after tumor resection[380]. Similar findings have been reported in prospective studies of HCC in alcoholic cirrhosis[241] and in HCV-associated cirrhosis[381].

Several studies suggest an association between HCC and dietary iron overload from beer fermented in steel drums in black Africans[382-385]. Furthermore, experimental evidence has identified several mechanisms of iron involvement in HCC development. Namely, HCC cells, like many other cancer cells, upregulate iron uptake and intracellular iron accumulation since they are dependent on iron[386,387]. The generation of ROS by this iron favors carcinogenesis through promotion of genomic instability and generation of DNA repair defects[388,389]; in other words,, this generation of ROS maintains the oncogenic phenotype of cancer cells[390,391]. The direct hepatocarcinogenic effect of free iron in the pathogenesis of HCC has also been demonstrated in an animal model of iron-rich diet where the tumor developed without fibrosis or cirrhosis[392,393]. Additionally, iron deposition directly decreases p53 protein level and its activity in the liver, facilitating the development of HCC[394]. An important mediator of intracellular iron is the protein leucine-rich repeat protein 5 (FBXL5); exposure of FBXL5

knockout animals to chemical or viral carcinogens has been shown to result in increased liver tumor formation. More importantly, low levels of FBXL5 in HCC patients are associated with a poor prognosis [395]. Ferritin heavy chain (FTH) acts as a protector of HCC cells, increasing their cellular resistance to ferroptosis, thereby acting as an oncogene in the pathogenesis and progression of HCC[396].

HCC patients in contrast to those with other cancers have low hepcidin levels[397-399]. Many mechanisms lead to the final decrease of hepcidin in HCC, including downregulation of inducers such as HAMP, TfR and HJV, and upregulation of suppressors such as matriptase 2 and GDF15[400]. Hepcidin downregulation increases cellular proliferation and HCC risk *via* reduction of the hepcidin protection against HSC activation. The downregulation of hepcidin in HCC has been attributed to the effects of cirrhosis rather than to HCC itself. Cirrhotic patients also show decreased hepcidin expression irrespective of disease etiology[152,336,399], while the hepcidin:ferritin ratio has been reported to decrease with fibrosis progression[373].

Ferroptosis and its inducers have been extensively investigated in HCC as it is considered an effective tumor suppression mechanism[81,401-403]. On the other hand, genes negatively regulating ferroptosis increase HCC drug resistance[404]. Sorafenib, a drug used for treatment of advanced HCC, is one example. This drug can induce the expression of metallothionein-1G (MT-1G), and upregulation of MT-1G has been demonstrated to serve as a negative regulator of ferroptosis, conferring resistance to sorafenib[405]. Some studies have also found that haloperidol can facilitate the cascade of ferroptosis induced by sorafenib in HCC[406].

In contrast to the negative regulators of ferroptosis, ACSL4 can positively regulate ferroptosis in HCC [97]. Inhibition of ACSL4 protects sorafenib-induced ferroptosis in HCC cells. A human study demonstrated an upregulation of the ACSL4 protein in HCC tissue from surgical specimens with a good response to sorafenib as a postsurgical adjunct treatment[407]. ACSL4 may therefore serve as a prognostic factor for survival and disease-free survival time[407,408].

Natural omega-3 PUFAs are the main peroxide substrates in ferroptosis and have anti-tumor activity [409], a fact that has been therapeutically exploited[410]. PUFAs consumed in the form of fish can reduce the risk of HCC development[411]. Ceruloplasmin has also been shown to inhibit ferroptosis in HCC cells, interfering with iron metabolism. Moreover, inhibition of ceruloplasmin increases the accumulation of iron and ROS production, facilitating erastin-induced ferroptosis in HCC cells[412].

Additional regulators of ferroptosis in HCC are the long non coding RNA molecules (lncRNAs), but their role has not been fully elucidated[413]. Erastin-induced ferroptosis upregulates the lncRNA GABPB1-AS1 in HepG2 cells, silencing the gene encoding peroxiredoxin-5 peroxidase and eventually leading to a reduction in cellular antioxidant capacity[414]. The predictive value of lncRNAs associated with ferroptosis in HCC has been recently addressed. Nine and five ferroptosis signature models have been established, which identified two groups of patients; the high-risk group in this study was shown to have enhanced tumorigenesis and worse prognosis[415,416].

Equally, the non-coding circular RNAs (circRNAs) seem to play a role in the development of HCC through ferroptosis. The circ0097009 endogenous RNA regulates the expression of SLC7A11, a key regulator of cancer cell ferroptosis in HCC. Circ0097009 therefore may be used as a potential target for HCC treatment[417].

Ferroptosis-related genes (FRGs) have also been identified and found to be upregulated in HCC tissue. In one study, three clusters have been determined, and a high expression of cluster 3 has been associated with worse prognosis and a higher histological stage[418]. Another approach regarding the use of ferroptosis as a prognostic marker in HCC has also recently been presented in which a novel ferroptosis-related 10-gene signature stratified HCC patients into two risk groups[419]. Those in the high-risk group have significantly reduced survival. The role of ferroptosis in HCC generation and progress has been recently reviewed[420].

CHOLESTATIC DISEASES

Hepcidin is significantly lower in patients with primary biliary cholangitis and primary sclerosing cholangitis compared to patients with other chronic viral and metabolic liver diseases. In one study, low hepcidin was maintained even after two years of treatment[421]. The reason for low hepcidin may be the suppression of STAT3 phosphorylation by accumulated bile acids. Furthermore, hepcidin remains lower in cholestatic cirrhosis compared to non-cholestatic cirrhosis, suggesting the critical role of cholestasis in maintaining low values of hepcidin[422].

AUTOIMMUNE HEPATITIS

There is experimental evidence suggesting that iron is implicated in autoimmune hepatitis (AIH) through ferroptosis involvement. The classical AIH-inducer Concanavalin A (ConA) has been linked to an overproduction of reactive nitrogen species (RNS) such as nitric oxide and peroxynitrite in a mouse model of AIH. This effect is attenuated by Fer-1, indicating that ConA induces ferroptosis in the liver.

Moreover, gadolinium chloride (a Kupffer cell depleting agent) inhibits RNS and hepatocyte ferroptosis [423]. Indoleamine 2,3-dioxygenase 1 (IDO1) is an intracellular heme enzyme involved in autoimmune diseases[424]. Upregulation of IDO1 has also been shown to be involved in ConA-induced hepatocyte ferroptosis through RNS accumulation and hepatocyte ferroptosis. An IDO1 inhibitor and an IDO1 knockout were shown to induce this effect, indicating that IDO1 promotes hepatocyte ferroptosis by triggering nitrative stress[425].

Clinical evidence also supports the detrimental effect of iron in AIH. Ferritin and iron are increased in serum of 65% and 58% of naïve patients with AIH respectively, which is resolved after successful treatment[426]. Increased serum ferritin has been independently associated with advanced fibrosis in patients with untreated AIH[427]. Moreover, serum hepcidin is low in patients with liver autoimmune disease[367,421]. Interestingly, in AIH, low serum hepcidin levels remain after 2 years of treatment, a finding similar to observations in autoimmune cholestasis. A plausible explanation could be that hepcidin is involved in hepatic autoimmune processes[428].

ISCHEMIA-REPERFUSION INJURY

Although ischemia-reperfusion injury (IRI) is not strictly a liver disease as it also occurs with other organ transplantations, iron is clearly involved in the pathogenesis of IRI-related hepatic abnormalities. Ferroptosis is implicated in the pathogenesis of IRI through GPX4 inactivation[59,429]. Iron overload and upregulation of the ferroptosis indicator PTGS2 are prominent characteristics of IRI in the liver[59]. An analysis of 202 live-donor liver transplantation patients showed a high serum ferritin level indicating iron overload[430]. In this study, use of ferroptosis inhibitors such as Fer-1, α-tocopherol, and DFO prevented hepatic IRI.

ACUTE LIVER FAILURE

Ferroptosis is also involved in the development of acute liver failure (ALF). In sepsis-induced ALF, analysis of the liver infiltrate has shown that FRGs may be responsible for the development of liver failure through the activities of B cells and natural killer cells[431]. The most common reason for ALF, however, is acetaminophen (APAP) toxicity in which lipid peroxidation leads to hepatocyte ferroptosis [432].

GSH is important for the inactivation of the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) responsible for APAP toxicity. GSH reduction and GPX4 inhibition are common in APAP-induced cell death[433]. The viability of mouse hepatocytes in the presence of APAP is improved by fer-1 without restoring the cellular GSH level, suggesting that suppression of the conversion of APAP to NAPQI is not the reason for the protective effect of fer-1[434]. Consistently, other experiments have confirmed the role of ferroptosis in APAP-induced hepatocyte cell death[432,435-437]. An additional mechanism of APAP-induced ferroptosis is the significant hepcidin reduction, likely *via* activation of HIF1α[434,438-440].

However, the role of ferroptosis in APAP toxicity and other drug-induced liver injury is disputed. An earlier report showed that α-tocopherol does not improve APAP-induced liver injury and that lipid peroxidation is not involved in APAP hepatotoxicity[441]. A recent review suggests that APAP-induced hepatotoxicity should be identified as programmed necrosis and not ferroptosis or other types of cell death[442]. Therefore, more research is required before ferroptosis inhibitors are recommended as treatments for APAP toxicity.

SICKLE CELL LIVER DISEASE

Sickle cell liver disease (SCD) is an inherited disease caused by the presence of hemoglobin S. Under hypoxic conditions, red blood cells are dehydrated and form the characteristic sickle cells[443,444]. The formation of hemoglobin S is due to a single substitution of an amino (glutamic acid to valine) in the beta globin chain[444]. Viral hepatitis and iron overload are two major reasons for the development of liver disease in SCD, both of which are typically related to patients receiving multiple blood transfusions[445]. Sources of hepatic iron in SCD include these multiple blood transfusions and chronic intravascular hemolysis[446]. Liver iron deposition occurs mainly in Kupffer cells[447]. Liver iron deposition can also occur in non-transfusion dependent patients[448], and there is a single case described in a patient who never received any blood transfusion[449]. Hemosiderosis in SCD may lead to fibrosis and overt cirrhosis[445,448,449].

CORONAVIRUS DISEASE 2019

There is considerable evidence to suggest an association between ferroptosis and coronavirus disease 2019. Cytokines produced during the infection have been shown to upregulate hepcidin expression, which leads to ferroportin suppression and iron accumulation. In addition, severe acute respiratory disease coronavirus 2 downregulates the expression of GPX4, contributing further to the initiation of the Fenton reaction and production of massive amounts of ROS and associated ferroptosis[450].

TARGETING IRON

There have been many attempts to reduce iron overload, which is uniformly considered detrimental in liver disease irrespective of etiology. However, it should be remembered that iron loading is not always similar between patients and between stages of various diseases[152]. Dietary iron restriction has been shown to be effective in reducing liver fibrosis and steatosis in diet-induced NAFLD animal models[451,452].

Phlebotomy is the traditional treatment in hereditary hemochromatosis, as it increases erythropoiesis, partially reverses liver fibrosis, and increases life expectancy[453,454]. Phlebotomy has been used to treat NASH patients, but the clinical benefit is unclear[455]. Phlebotomy improves liver enzymes, insulin resistance, and liver histology in the majority of NAFLD patients, but it is not fully successful in DIOS insulin resistant patients with slight ferritin increase[182,456-458]. Insulin sensitivity is improved by phlebotomy in type II diabetics with a high serum ferritin[459]. Moreover, in patients with the metabolic syndrome, phlebotomy improves metabolic parameters, including glycosylated hemoglobin A1c and LDL/high-density lipoprotein ratio[460]. In a meta-analysis of four interventional studies with more than 400 patients, phlebotomy was shown to improve liver enzymes, insulin resistance, and lipid abnormalities[461].

In contrast, no effect was reported in two prospective randomized controlled trials. The first, which is the largest series so far, was conducted in NAFLD patients[462], and the second in DIOS patients with insulin resistance[463]. To this end, the benefit of phlebotomy in patients with NASH remains unclear until more extensive studies are available[464].

Phlebotomy reduces the marker of oxidative stress 8-hydroxy-2′-deoxyguanosine in HCV patients who have failed IFN therapy. Fibrosis and inflammation are also reduced, but HCV titers are unaffected. None of the patients in these studies were shown to develop HCC at the six year follow-up point[306,465]. Reduction of HCC development in HCV patients after phlebotomy has been verified in additional studies[466,467]. Phlebotomy has also been reported to improve the response to IFN in chronic HCV[468].

Iron chelation is an additional intervention to reduce liver iron. DFO has been successfully used to control fibrosis in hemochromatosis[469]. Studies in several animal models have revealed that iron chelation decreases the stability of procollagen mRNA[470] and reduces elastin mRNA[194]. DFO has also been shown to reverse HSC activation and induces apoptosis of activated murine HSCs[471]. More recently, a study of the combination of DFO with pegylated IFN-α showed a synergistic anti-fibrotic effect in rats[472]. ROS degrade the apolipoprotein B100 (apoB100) component of VLDL, thereby enhancing hepatocyte steatosis in rodents. In another study, DFO restored apoB100 and increased VLDL secretion[473]. No firm conclusions can be drawn, however, without the results of clinical trials. It should be noted that inhibition of hemoxygenase-1 decreases hepatic iron deposition and attenuates liver fibrosis in rats[474].

Interestingly, commonly used drugs like the calcium channel blockers have been found to induce HSC apoptosis and reduce DMT1 expression, hepatic iron deposition, and liver collagen in mouse and cellular experiments[475]. Hepcidin may be a promising agent for the treatment of liver iron overload, as hepcidin administration has been shown to attenuate iron deposition in mouse models of hemochromatosis[476-478], while its overexpression ameliorates fibrosis severity. This is due to the inhibition by hepcidin of the TGFβ1-induced SMAD3 phosphorylation in HSCs, a pathway that requires the presence of ferroportin in stellate cells[357]. Similar reduction of liver fibrosis has been observed with BMP6 overexpression in murine and human NAFLD[358].

Hepcidin responds to iron conditions in HCV patients, but the response is impaired. Thus, correction of hepcidin regulation may improve the clinical progress in iron-overloaded HCV patients[479]. Hepcidin manipulation may be beneficial in the management of HCC as well. The iron chelator deferasirox induces apoptosis in hepatoma cells lines and decreases liver tumor development in mice, increasing HAMP mRNA expression. However, toxicity and the lack of response in some patients may be a problem in human trials[480]. Additionally, some HCC patients have increased hepcidin expression and downregulation of hepcidin may be required. In a murine HCC model with high liver hepcidin, the traditional Chinese medicinal herb dandelion polysaccharide has been shown to reduce hepcidin expression, arrest the cell cycle, and suppress the HCC proliferation[481]. Hepcidin, therefore, is a logical candidate target for clinical trials in HCC. Indeed, both hepcidin agonists and inhibitors have been tested *in vitro* and in laboratory animals[482]. It should be noted that synthetic mini-hepcidins have

also been tested in Hamp -/- mice; in one study, serum iron was reduced after chronic administration of the drug[476].

Ferroptosis is the current therapeutic target in the treatment of iron overload diseases. It should be stressed, however, that the effects of ferroptosis in chronic liver disease depends on the cell type and the specific environment. In liver fibrosis, for example, ferroptosis has different effects on hepatocytes and HSCs as will be detailed later[483]. A future challenge is to develop drug delivery systems targeting ferroptosis in specific cell types. In ALD and in NAFLD, ferroptosis is implicated in liver damage, and ferroptosis inhibition would theoretically be beneficial[225,276,432]. For example, ferroptosis-induced liver injury could be reversed by sestrin 2, an antioxidant protein increased by ferroptosis inducers[484].

Ferroptosis inducers

In contrast to other liver diseases where ferroptosis is detrimental and therapies are directed towards inhibition of ferroptosis, HCC is benefited by enhancement of ferroptosis. Thus, ferroptosis inducers are used in advanced HCC. Sorafenib, a multi-kinase inhibitor, is the most extensively studied ferroptosis inducer[103,485]. In HCC, this drug acts by inhibiting cellular proliferation and neo-angiogenesis. Additionally, it induces ferroptosis in HCC cells[486]. It has been reported that sorafenib decreases the uptake of cystine in the Xc- system and starts the chain of events leading to ferroptosis induction through the accumulation of ROS, which is the result of GSH depletion and loss of GXP4 activity[487]. Excessive ROS production also results in the inhibition of the retinoblastoma protein Rb, an important negative regulator of cell proliferation[488].

Prolonged administration increases the resistance of HCC cells to sorafenib. ABCC5, a recently described regulator of ferroptosis, increases the generation of GSH and reduces the production of ROS through stabilization of SLCA11 and subsequent inhibition of ferroptosis. Accordingly, downregulation of ACCC5 reduces resistance to sorafenib[489]. Other proteins reducing the sorafenib-induced ferroptosis through stabilization of SLCA11 have also been recently described[490,491].

Haloperidol has also been shown to promote erastin- and sorafenib-induced ferroptosis, suggesting that it could be used in combination with sorafenib to achieve either dosage or resistance reduction[404,406,492]. An upregulation of Nrf2 through activation of the p62-Keap1-Nrf2 pathway inhibits sorafenib-induced ferroptosis in HCC cell lines[63,493]. Interestingly, trigonelline, the active ingredient of the traditional Chinese medicine fenugreek, increases ferroptosis by acting on Nrf2, therefore reducing sorafenib resistance[494]. Overexpression of the leukemia inhibitory factor receptor (LIFR) has also been shown to increase sorafenib-induced ferroptosis of HCC cell lines, whereas reduced LIFR expression increases resistance to ferroptosis[495].

A recent study reported an another target for HCC treatment. In this study, lactate-rich hepatoma cells were shown to exhibit increased resistance to the ferroptosis generated by common ferroptosis inducers. Moreover, lactate uptake was shown to be mediated by monocarboxylate transporter 1 (MCT1), which enhances the production of monounsaturated fatty acids, blocking ferroptosis. Inhibition of MCT1-mediated lactate uptake enhances ferroptosis[496]. In contrast to the presented evidence, a recent report indicated that sorafenib may not be an inducer of ferroptosis at least in many cancer cell lines[497]. Other drugs that could be used in the treatment of HCC based on increased ferroptosis have also been recently described[498,499]. Heteronemin, a marine terpenoid, induces ferroptosis in HCC cells by reducing GPX4[500]. IFN-γ has also been confirmed to inhibit system Xc- activity and increase ferroptosis[501]. Lenvatinib, another kinase inhibitor used in advanced HCC treatment, also acts through the inhibition of the system Xc-. Fibroblast growth factor receptor-4 (FGFR4) increases the activity of the system Xc- and lenvatinib inhibited FGFR4 increasing ferroptosis. Interestingly, patients with HCC positive for FGFR4 have a longer progression-free survival compared to those with FGFR4-negative HCC. Nrf2 upregulation has also been shown to decrease the sensitivity of HCC to lenvatinib [502]. Moreover, low-density lipoprotein nanoparticles (LDL-DHA NPs), selectively induce HCC cell death in mouse models, and LDL-DHA NPs enhance lipid peroxidation due to both GSH depletion (leading to GPX4 inactivation) and direct degradation[410].

Ferroptosis can be used for stratification of HCC patients to predict both prognosis and suitability for immunotherapy. For that purpose, a ferroptosis-related prognosis risk score model has been developed to stratify patients into two subgroups based on six FRGs (FRGs)[503].

Ferroptosis inhibitors are promising drugs in the treatment of various liver diseases, although evidence is mainly based on laboratory data. NAFLD and NASH progress is worsened by induction of ferroptosis[98,224,504]. Alleviation of NASH can be achieved by ferroptosis inhibitors, such as liproxstatin-1 or ferrostatin-1[225,505]. In one study, administration of the ferroptosis inducer RSL3 aggravated hepatic steatosis and inflammation in diet-induced NASH mice, while administration of liproxstatin-1 ameliorated NASH severity and rescued animals from cell death[225].

Other drugs, such as Ginkgolide B and dehydroabietic acid, alleviate NASH severity by inhibiting ferroptosis *via* upregulation of the p62-Keap1-Nrf2 pathway[506-508]. Thymosin β4 (Tβ4) improves liver lipid metabolism markers in NAFLD rat models and inhibits the palmitic acid-induced hepatocyte death in the LO2 cell line. Ferrostatin-1 increases the effect of Tβ4, which is attenuated by erastin, indicating that the protection of hepatocytes is mediated by ferroptosis reduction[509]. The enzyme enoyl coenzyme A hydratase 1 (ECH1) is an important component of mitochondrial fatty acid β-oxidation. ECH1 knockdown aggravates liver inflammation and fibrosis in mouse NAFLD models

while fer-1 administration alleviates liver damage, again suggesting that the beneficial effect of ECH1 may be due to inhibition of ferroptosis[505].

Liver fibrosis is another disease that may be treated by ferroptosis regulators[510]. Inhibition of ferroptosis by ferrostatin 1 reverses liver fibrosis induced by a high-iron diet or and carbon tetrachloride [511], while induction of ferroptosis by liver iron overload aggravates APAP-induced fibrosis in mice [483]. However, ferroptosis is a double-edged sword in liver fibrosis. When ferroptosis is targeting activated HSCs, the induction of ferroptosis is beneficial. The cystine/glutamate antiporter SLCA11 has been shown to increase ferroptosis as mentioned before[55]. Inhibition of SLC7A11 enhances ferroptosis in HSCs and attenuates liver fibrosis[512]. Likewise, erastin and sorafenib induce ferroptosis in HSCs, and reduced liver fibrosis in mice[371,513].

There is growing evidence that natural products may effectively be used in the treatment of liver fibrosis. Artesunate can attenuate liver fibrosis by triggering ferritinophagy-mediated ferroptosis in HSCs[121]. Artemether can also induce ferroptosis in HSCs by increasing iron and ROS in HSCs[514] and promoting p53-dependent ferroptosis[372]. MgIG can also induce ferroptosis in HSCs by increasing the activity of the enzyme HO-1[123].

Chrysophanol isolated from the rhizome of rhubarb can inhibit the HBV x protein-induced activation of HSCs through ferroptosis and alleviate HBV-related fibrosis[515]. Additionally, wild bitter melon extracts can downregulate GPX4 and SLC7A11 in activated HSCs by inducing ferroptosis[516]. Two other proteins regulating ferroptosis in HSCs could be the future targets in the treatment of liver fibrosis: ZFP36/TTP and ELAVL1/HuR. These are critical regulators of HSCs ferroptosis[371,513]; ZFP36 protects against ferroptosis and ELAVL1 contributes to ferroptotic cell death.

Three more diseases may be benefited from ferroptosis inhibitors. Fer-1 improves I/R-mediated liver disease[59,224,276]. ALF is also a candidate for similar treatment based on experimental data. Glycyrrhizin, an active constituent of the licorice root, reduces ferroptosis during ALF, inhibiting oxidative stress through the Nrf2/HO-1/high mobility group box 1 pathway[517]. Finally, reduction of liver iron load will most certainly benefit ALD. Phlebotomy, however, is not recommended in patients with ALD.

An interesting approach to reduce iron load in ALD is the stabilization of erythrocytes and associated reduction in hemolysis. Administration of N-acetylcysteine or protective heme carriers like haptoglobin and hemopexin has been tested. Erythrocyte stabilizers include vitamins such as B12 or folate[281]. Ferrostatin-1 can also reduce alcoholic liver damage[276], indicating participation of ferroptosis in ALD progression. Dimethylfumarate reduces lipid peroxidation and alleviates liver cell ferroptosis leading to ALD improvement in a murine model[275]. Currently, no effective treatment can be recommended for ethanol-induced iron overload. Modulation of ferroptosis for the treatment of chronic liver diseases has been recently reviewed[282].

NUTRIENTS AS TREATMENT OPTIONS OF LIVER IRON OVERLOAD

Vitamin A

Retinoid signaling is decreased in the livers of humans and mice with NAFLD[518,519], and is epigenetically silenced in HCC[520]. Administration of the synthetic retinoid tamibarotene improved oxidative stress and iron deposition in iron-fed mice. Retinoids downregulate the hepatic expression of HJV, leading to liver hepcidin downregulation and ferroportin upregulation[521,522]. Retinoids also attenuate insulin resistance and hepatic steatosis in a murine model of NAFLD[523,524]. Attenuation of hyperinsulinemia may prevent the development of HCC in NAFLD[525].

Vitamin C

A very large observational study with more than 8000 participants demonstrated that dietary vitamin C supplementation decreases plasma ferritin levels[526], indicating that vitamin C limits iron deposition and thereby increases iron mobilization. In a murine model of ALD, vitamin C administration was shown to restore hepatic hepcidin and downregulate intestinal ferroportin, leading to HIO amelioration [527]. Therefore, it is reasonable to supplement vitamin C in ALD and chronic HCV patients with hepatic iron deposition.

Vitamin D

Evidence from patients with thalassemia major and hereditary hemochromatosis indicates that iron overload suppresses vitamin D, as there is a negative correlation between liver iron and 25-hydroxyvitamin D levels[528-530]. In hereditary hemochromatosis, levels of vitamin D are partially restored after phlebotomy[531]. Moreover, vitamin D depletion exacerbates HIO in HJV knockout mice, an effect that is corrected by the administration of the calcium channel blocker verapamil but not by vitamin D supplementation[475,532]. These results indicate a link between iron and calcium and justify the use of calcium channel blockers as a treatment modality for iron deposition in patients with decreased levels of vitamin D, as is frequently observed in ALD, NAFLD, and chronic HCV[533-536].

Zinc

Zinc-deficient diet has been shown to lead to increased plasma ferritin and development of HIO in rats, while zinc supplementation returns liver iron to normal[537]. Clinical studies also indicate that iron deficiency anemia is frequently associated with zinc deficiency[538,539], implying a physiological crosstalk between iron and zinc. For example, zinc plus iron administration in rats has been demonstrated to ameliorate anemia more efficiently than iron alone[540]. The therapeutic effects of zinc on chronic liver diseases have been reviewed[541].

Folate

The Solute Carrier Family 46 Member 1 (SLC46A1) is the major importer of hemeiron in the duodenum, and it is also present in the liver. In murine liver-specific SLC46A1 knockdowns, its role in liver iron deposition has been investigated. In these studies, SLC46A1 was found to take up heme in the liver and contribute to hepatic iron deposition. Interestingly, heme inhibited folate uptake after downregulation of SLC46A1 expression, but folate supplementation had no effect in heme uptake and SLC46A1 expression indicating that folate deficiency was the result of secondary liver heme uptake excess[542]. Accordingly, the combined administration of iron and folate in rats also significantly reduced liver iron compared with iron alone[543].

Riboflavin

Contrary to the agonistic use of the previously discussed nutrients in the treatment of liver iron deposition, riboflavin antagonists, such as galactoflavin, may be used in HIO[544]. This is because riboflavin deficiency leads to a decrease in iron absorption[544,545]. A detailed discussion on the role of nutrients in chronic liver diseases was recently published[546].

CONCLUSION

It is well documented, that iron in the liver is a double-edged sword. It is a necessary element in many metabolic pathways, but it is equally harmful if either the amount or its cellular localization are unbalanced. Increased iron deposition negatively affects most chronic liver diseases. The interplay with lipid metabolism prompted an extensive investigation for the role of iron in NAFLD/NASH. Moreover, the description of ferroptosis as a discrete form of regulated hepatocyte death opened the way for the therapeutic modulation of iron overload in many diseases. Interestingly, most liver diseases are benefited by ferroptosis inhibition. A notable exception is HCC, where the therapeutic target is ferroptosis induction.

The current evidence involves the integration of information from experimental models and less so, from patient findings. Further experimental *in vitro* and *in vivo* investigations are warranted to find more suitable molecules with wider availability and better specificity that could regulate ferroptosis. In this context, it is interesting to note that many natural products may influence iron metabolism and ferroptosis. Furthermore, it should be stressed that clinical trials involving ferroptosis regulation are scarce and sometimes inconclusive. Therefore, to draw valid conclusions, further well-designed randomized trials in humans are urgently required.

FOOTNOTES

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Country/Territory of origin: Greece

ORCID number: Elias Kouroumalis 0000-0002-6875-906X; Ioannis Tsomidis 0000-0002-8595-2750; Argyro Voumvouraki 0000-0002-2725-6028.

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MINIREVIEWS

COVID-19 and the liver: Are footprints still there?

Tarana Gupta, Hemant Sharma

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Tarana Gupta, Hemant Sharma, Department of Medicine, Pandit Bhagwat Dayal Sharma Post Graduate Institute of Medical Sciences, Rohtak 124001, Haryana, India

Corresponding author: Tarana Gupta, Doctor, MBBS, MD, Professor, Department of Medicine, Pandit Bhagwat Dayal Sharma Post Graduate Institute of Medical Sciences, Medical Mor, Rohtak 124001, Haryana, India. taranagupta@gmail.com

Abstract

The coronavirus disease 2019 (COVID-19) hit the entire world as a global pandemic and soon became the most important concern for all patients with chronic diseases. An early trend in higher mortality in patients with acute respiratory distress attracted all researchers to closely monitor patients for the involvement of other systems. It soon became apparent that patients with chronic liver diseases are at increased risk of mortality given their cirrhosis-associated immune dysfunction. Additionally, liver function abnormalities were noted in patients with severe COVID-19. Profound cytokine storm, direct viral infection, drugs and reactivation of viral infections were causes of deranged liver functions. Here, we discuss the relation between COVID-19 and chronic liver disease, specifically cirrhosis, hepatitis B, hepatitis C, and non-alcoholic fatty liver disease (NAFLD), as well as the liver manifestations of COVID-19. The metabolic syndrome, obesity, diabetes mellitus and NAFLD were found to worsen outcome in different studies reported worldwide. Decompensated cirrhosis should be considered a risk factor for death and severe COVID-19. Recently, COVID-19 related cholangiopathy has also been reported with changes of secondary sclerosing cholangitis. The long-term persistence of viral antigens in gut epithelia raises concern regarding the future risk of autoimmune liver diseases.

Key Words: COVID-19; Chronic liver disease; Cirrhosis; Liver injury; Transaminases; Non-alcoholic fatty liver disease; Post-acute COVID-19 syndrome

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Core Tip: Coronavirus disease 2019 (COVID-19) and liver involvement have been a major concern since the beginning of the COVID-19 pandemic. Deranged liver functions with raised transaminases were reported in patients with severe COVID-19. On the other hand, acute hepatitis or liver failure was uncommon. Severe acute respiratory syndrome coronavirus 2 virus associated cytokine surge, systemic inflammation, direct viral infection, drugs such as remdesivir, steroids, and lopinavir-ritonavir were the main causative factor in raised transaminases. Patients with pre-existing chronic liver diseases especially non-alcoholic fatty liver disease were found to be risk factors for increased mortality in patients with severe COVID-19.

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INTRODUCTION

In December 2019, severe acute respiratory syndrome (SARS) caused by SARS- coronavirus 2 (SARS-CoV2), which belongs to the Coronaviridae family, was first detected in Wuhan, China. It soon spread to the rest of the world, and was declared a global pandemic in March 2020. In mild cases, the symptoms included fever, cough, body aches, malaise, loss of taste and smell. Approximately 15% of patients would eventually have respiratory compromise, hypoxia, and the need for invasive mechanical ventilation. Finally, multi-organ failure, coagulopathy, disseminated intravascular coagulation, acute respiratory distress syndrome, and hypoxia would follow. Over a period of more than 2 years, multiple waves of coronavirus disease 2019 (COVID-19) were observed in different geographical regions. As the virus mutated, there were many shifts in the clinical presentation. New symptoms of predominantly the upper respiratory tract such as sneezing and rhinitis, gastrointestinal symptoms such as diarrhea and non-specific abdominal pain, cardiac symptoms such as arrhythmias, ocular and neurological symptoms were reported. Additionally, as patients underwent more investigations, dysregulated coagulation and thrombosis were documented. Overall, liver involvement such as elevated liver enzymes ranged from 14% to 53% of patients in various studies. However, acute hepatitis or liver failure was uncommon. Furthermore, different studies worldwide have shown that non-alcoholic fatty liver disease (NAFLD), diabetes, hypertension, and obesity are significant risk factors for severe COVID-19.

A lockdown and a ban on air travel during the COVID-19 pandemic helped keep people with chronic illnesses at home. Their overall exposure to COVID-19 and other pathogens was constrained. Mild COVID-19 patients were isolated and quarantined in accordance with protocol and did not frequently undergo examinations. Patients with serious illnesses, however, were the only ones who underwent in-depth examinations. As a result, the majority of research mainly included individuals with serious diseases.

METHODS

We searched PubMed, Google Scholar, and Google from January 2020 to August 2022, for articles written in English that describe the liver effects of COVID-19, using the search terms "coronaviruses and liver", "COVID-19 and liver", "COVID-19 and liver symptoms", "COVID-19 and hepatic", "COVID-19 and liver function test", "COVID-19 and liver inflammation", "SARS-CoV-2 and liver", "COVID-19 and NAFLD", "COVID-19 and non-alcoholic fatty liver disease", "COVID-19 and non-alcoholic fatty liver disease", "COVID-19 and hepatitis", and "COVID-19 and Vaccine". Reference lists of the articles were scanned to identify any additional studies. The title and abstract of each article were read for the initial selection and then the full-text articles were read on availability. Reference lists of the full-text articles were scanned to identify any additional studies. All types of research articles, including original research articles, reviews, case series, short communications, and case reports were considered. Of the 667 articles identified, 313 were studied for this review.

SARS-COV2 HEPATOTROPISM

Due to a lack of significant laboratory testing and tissue biopsies from patients who were actively infected with SARS-CoV2, the mechanism of its replication is still not entirely understood.

SARS-CoV2 is an enveloped positive sense single stranded RNA virus with almost 80% identity with SARS-CoV. It has 4 structural proteins namely nucleocapsid, spike (S), membrane and enveloped proteins. The spike protein has multiple protrusions from the cell surface giving the virus its appearance and name. The angiotensin-converting enzyme 2 (ACE) receptors are the potential site of entry for SARS-CoV2. ACE2 receptors are abundantly present on alveolar epithelium, lung, nasal epithelium *etc.* They are also present in fewer numbers in intestinal epithelium and liver[1]. The spike protein having two subunits S1 and S2 interacts with the ACE2 receptor for virus entry. However, ACE2 receptors are not sufficient alone and transmembrane serine proteases 2 (TMPRSS2) in addition to basic amino acid cleaving enzymes (FURIN) are essential for virus entry. According to single cell RNA sequencing analysis, hepatocytes have less co-expression of TMPRSS2 and ACE2 receptors. In the liver, cholangiocytes and sinusoidal endothelial cells have the highest expression of the ACE2 gene in almost 60% of the cell population as compared to hepatocytes (3% cells)[2,3]. Thus, a tissue or organoid model is required to understand the permissibility of liver cell types to SARS CoV-2 infection. Zhao *et al*[4] created human liver ductal organoids that were able to replicate SARS-CoV2 infection and expressed ACE2 and TMPRSS2. This suggests that the bile duct epithelium may be able to support pseudoparticle invasion. Despite a higher number of SARS-CoV2 virus receptors and a higher risk of infection of bile duct epithelia, COVID-19 does not follow a cholestatic pattern[5].

Studies conducted before the COVID-19 pandemic indicated that patients with hepatitis C virus (HCV)-related cirrhosis had 30 times higher ACE2 receptor expression on hepatocytes than healthy individuals[6]. The overexpression of ACE2 and TMPRSS2 has also been documented in obesity and nonalcoholic steatohepatitis patients, but not in patients with steatosis alone[7]. ACE2 is an interferon-inducible gene found in human respiratory epithelia, possibly SARS-CoV2 hepatotropism can be potentiated by the effects of systemic inflammation on hepatocytes and can lead to hepatocyte injury[8,9].

Additionally, ACE2 receptors are found in intestinal epithelia/enterocytes and SARS-CoV2 RNA has been documented by polymerase chain reaction in stool up to one week after recovery from respiratory illness. The latest data suggests that viral protein and RNA are found in intestinal biopsies for several months after resolution of respiratory illness[5,10].

In a study from Italy, postmortem wedged liver biopsy samples from 48 patients dying from severe COVID-19 were examined[11]. The results revealed vascular abnormalities such as sinusoidal and partial to complete portal venous microthromboses in almost 100% of samples. Additionally, mild portal inflammation, portal fibrosis, microvesicular and macrovesicular steatosis were documented in 66%, 60%, and 50% of patients, respectively. The latter finding is probably related to pre-existing liver disease such as NAFLD, as suggested by the presence of metabolic risk factors which were more prevalent in this patient group. Electron microscopy of these biopsies also revealed potential coronavirus-like particles, mitochondrial edema, and apoptosis of hepatocytes. However, comprehensive proteomic analysis of autopsy tissue from 19 patients with COVID-19 did not find signs of viral replication[12].

Furthermore, proteomic profiling revealed disrupted oxidative phosphorylation, fatty acid oxidation, and up-regulated immunological activators and profibrotic pathways. It is possible that hepatic steatosis, coagulative necrosis, and multi-organ dysfunction were all linked to mitochondrial dysfunction, dysregulated oxidative phosphorylation, *etc*[13].

LIVER FUNCTION TEST AND COVID-19

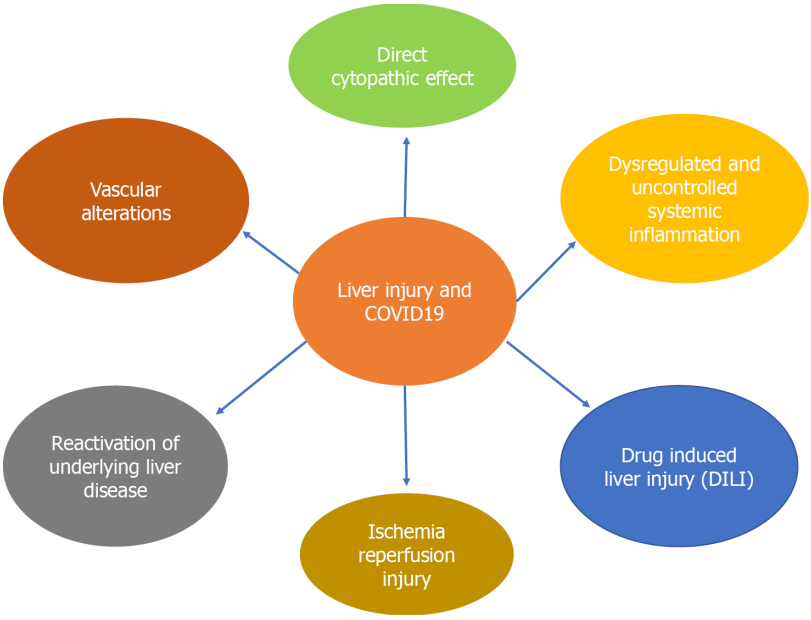
Despite higher SARS-CoV2 receptor expression on cholangiocytes and SECs, liver function derangement is usually in the form of a mild elevation in liver enzymes [1-2 upper limit of normal (ULN)][14-16].

Singh *et al*[17] showed that the presence of pre-existing liver illness has no effect on the incidence of liver enzyme elevations, although patients with pre-existing liver disease had a higher mortality rate.

In COVID-19, SARS-CoV2 induces a systemic inflammatory response and the release of cytokines. The predominant molecules are interleukin-6 and tumor necrosis factor alpha (TNF-alpha). Elevated cytokines result in hepatocyte inflammation and injury with liver ischemia, hypoxia, worsening of already existing chronic liver disease (CLD) and/or toxicity of medications used to treat the illness (Figure 1). Hepatic congestion as well as potential direct infection of hepatocytes although uncommon may also result in the release of transaminases[18].

However, indicators of muscle breakdown or systemic inflammation did not correlate with serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in hospitalised COVID-19 patients[19,20].

As AST level was frequently observed to surpass ALT level throughout the course of COVID-19, this was similar to patients with alcoholic liver disease, ischemic hepatitis and cirrhosis compared to a traditional hepatocellular pattern where ALT level is greater than AST[19]. Possibly, COVID-19 related mitochondrial dysfunction results in hepatic steatosis and altered hepatic perfusion is the result of sinusoidal microthrombosis[11,21-23].



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Figure 1 Effects of COVID-19 and liver injury interaction. COVID-19: Coronavirus disease 2019; DILI: Drug induced liver injury.

Respiratory epithelia involvement by SARS-CoV2 leads to defective oxygenation and the release of cytokines causes peripheral vasodilation and reduced tissue perfusion; the resultant perfusion and oxygenation defect causes systemic hypoxia which is a contributory factor in hepatocyte injury[24].

Early in the pandemic as no definitive treatment was available, experimental therapies in the form of drugs such as tocilizumab, remdesivir and lopinavir-ritonavir were used, which are known to cause hepatic injury[25-29]. Remdesivir was documented to cause elevations in liver enzymes in different studies[30,31]. Tocilizumab was well known for its risk of hepatitis B virus (HBV) reactivation and screening of hepatitis B and hepatitis C was advised before its use.

Ponziani *et al*[32] and Yip *et al*[33] showed that elevations in liver enzymes were associated with an increased incidence of shock, ICU admissions and invasive ventilation. However, these studies could be biased as hospitalized patients with severe disease undergo intensive monitoring of liver function (which increases the chances of detecting liver injury) as compared to home isolated patients with mild disease due to quarantine.

Some studies have suggested that there is no apparent correlation between liver function derangement and mortality[34,35]. Others have suggested an increased risk of death in patients with ALT levels > ULN[16,36,37].

According to Bangash *et al*[38], elevated liver transaminases linked to COVID-19 are more likely caused by severity of the disease, in which the host's reaction and iatrogenic factors such as medication and invasive ventilation cause bystander liver injury and thus explain its link to mortality in a manner similar to that of sepsis[38]. Because of this, clinicians must focus more on these factors than just elevated aminotransferases especially in patients with no pre-existing liver disease.

COVID-19 AND CLD

In the early days of the COVID-19 pandemic, the hepatology community worked fast to establish the risk of SARS-CoV2 acquisition and harmful COVID-19 outcome in pre-existing CLD. According to data from major case series and population-level electronic health records during the first global spike, patients with CLD were not overrepresented, indicating that these diseases did not make patients more susceptible to infection[15,39]. In fact, a significant North American study discovered that people with cirrhosis had a decreased probability of SARS-CoV2 positivity, probably due to improved awareness, testing, and patient adherence to public health recommendations for home isolation and quarantine. However, it is now evident that individuals with cirrhosis are more likely to experience negative COVID-19 outcomes after infection, including mortality. Multiple lines of evidence, such as findings from the international registries SECURE-Cirrhosis and COVID-Hep[40], sizable observational cohorts such as the COVID-Cirrhosis-CHESS group[41], and population-level data, have all been used to support this. These registries were created early in the pandemic and interestingly, due to the emergence of the new Delta and Omicron variants as well as the introduction of vaccines, the relation between COVID-19 and liver will continue to evolve.

In a large registry cohort of 729 patients from 29 countries, it was discovered that mortality in individuals with cirrhosis after SARS-CoV2 infection was 32% overall, with case fatality increasing gradually with each Child-Pugh (CP) class (CLD without cirrhosis: 8%, CP-A: 19%, CP-B: 35%, CP-C: 51%)[42]. The rates of invasive mechanical ventilation, renal replacement treatment, and intensive care unit (ICU) hospitalisation all showed similar stepwise trajectories. Additionally, after adjusting for age and comorbidities, patients with decompensated cirrhosis (CP-B and CP-C) had a considerably higher probability of dying than patients without cirrhosis who tested positive for SARS-CoV2. Reports of elevated COVID-19 mortality in cirrhosis have been confirmed in two Asian-only registries[43] and in numerous multicenter cohort studies conducted in various geographic locations[44-46]. Iavarone *et al* [44] observed a 30-d mortality of 30% in Northern Italy during the early stages of the pandemic, which was much greater than a historical cohort of patients with cirrhosis hospitalised with bacterial infection [44]. Decompensated cirrhosis was also reported as an independent risk factor of death in CLD patients across 21 North American institutions[45]. Additionally, individuals with hepatocellular carcinoma (HCC) had a seven-fold higher chance of dying from COVID-19 than cirrhotic patients without HCC, indicating that this population may be particularly vulnerable to the side effects of SARS-CoV2 infection. A retrospective French cohort of > 259000 COVID-19 inpatients, including > 15000 with pre-existing CLD, showed that patients with decompensated cirrhosis had a higher adjusted risk of COVID-19 mortality[47]. This was in contrast to the findings in a nationwide Swedish cohort, which failed to identify a connection between cirrhosis and COVID-19 related mortality[48]. Cirrhosis overall, and decompensated cirrhosis in particular, should be considered a risk factor for death and severe COVID-19.

There are various characteristics related to the clinical course of COVID-19 in cirrhotic individuals. First, up to 46% of patients can present with acute hepatic decompensation, usually with new or worsening ascites and/or hepatic encephalopathy (HE)[42]. This can occur between 20% and 58% of the time even in the absence of the usual COVID-19 respiratory symptoms[42,44]. Patients with CLD present with gastrointestinal symptoms more frequently than matched controls[42]. This is linked to a more severe disease trajectory[45], a phenomenon that is widespread in society[49] and is connected to increased intestinal permeability, electrolyte imbalance, and systemic inflammatory load, and is documented in up to 12% to 50%[42-44,46] of patients with COVID-19 and decompensated cirrhosis. In the context of COVID-19, a number of well-known prognostic scoring models have been used to assess cirrhosis, with the CLIF-C ACLF score and CLIF organ failure scores surpassing Model for End-stage Liver Disease, North American Consortium for the Study of End-stage Liver Disease, and CP scores in the international and Latin American cohorts, respectively[42,50]. Actually, the likelihood of recovery rapidly decreases as organ support requirements increase. For instance, patients with CP-C cirrhosis have a mere 21% probability of surviving if admitted to the ICU, and decreases to 10% if mechanical breathing is necessary[42].

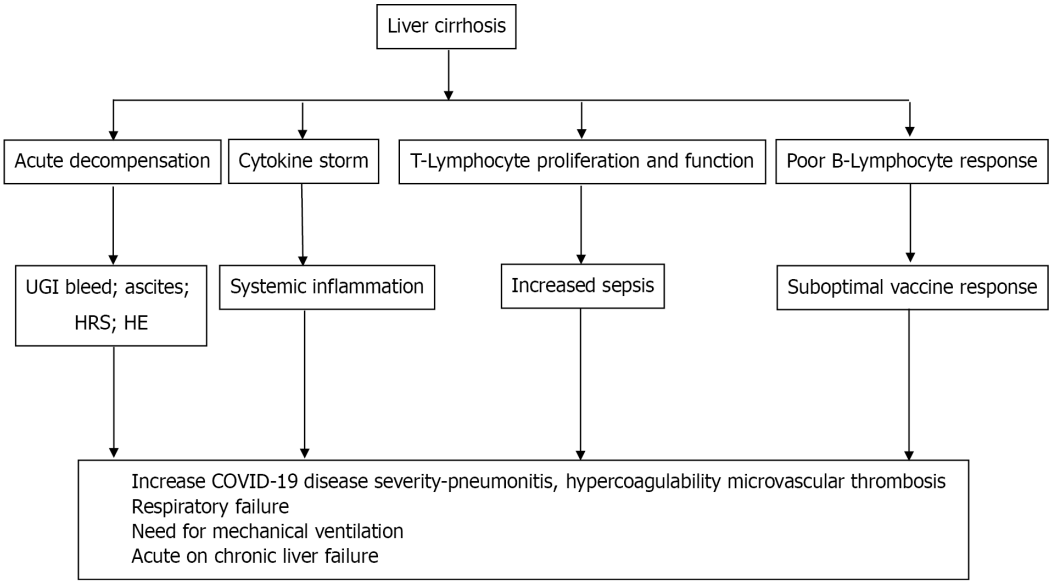
Despite the fact that SARS-CoV2 infection causes immediate hepatic decompensation, respiratory failure (71%) and problems related to the liver (19%) are the primary causes of death in individuals with cirrhosis[42]. Hepatic dysfunction and lung damage are likely linked by a number of overlapping pathways (Figure 2), including immunological dysfunction brought on by cirrhosis, coagulopathy, and altered pulmonary dynamics due to ascites and HE[51]. Given that the composition of the gut microbiota has been demonstrated to influence how the host immune system reacts to COVID-19, it is conceivable that intestinal permeability and dysbiosis linked to cirrhosis may also have a negative effect [52,53].

Although COVID-19 in patients with cirrhosis is linked to a significant immediate risk of death, rates of mortality and re-admission at 90 d appear equivalent to patients with cirrhosis alone in those who survive the initial shock[54]. Therefore, it appears that SARS-CoV2 infection does not accelerate the progression of liver disease beyond the course of cirrhosis after the acute infective period. However, up to 4 mo after recovering from acute COVID-19, hepatic MRI alterations, including enhanced T1 signaling, raised fat fraction, and hepatomegaly, have been found in 10% to 28% of otherwise healthy people[55,56]. In both patients with and without underlying CLD, it is unknown what these radiological characteristics following COVID-19 mean clinically long-term. Furthermore, although this remains unexplored and is not considered in the current investigations, these hepatic abnormalities might not be exclusive to COVID-19 and might also be present in individuals recovering from other severe systemic insults.

It is crucial to note that studies undertaken in the years before COVID-19 vaccination and the appearance of viral variants like Delta and Omicron are largely responsible for our knowledge of the disease course in individuals with COVID-19 and cirrhosis. CLD can affect 1% to 11% of people with SARS-CoV2 infection[57]. Numerous liver cirrhosis patients have been shown to have drunk alcohol in an ineffective effort to ward off coronavirus infection, raising the risk of alcoholic hepatitis[58].

Implications of COVID-19 include increased mortality associated with severe COVID-19, increased risk of hepatic decompensation, and decreased routine and HCC surveillance.

Although the acute mortality associated with COVID-19 in patients with cirrhosis is substantial, the rates of death and readmission at 90 d are equivalent to those in patients with cirrhosis alone in those who survived the initial insult[54]. Therefore, SARS-CoV2 infection does not appear to accelerate the course of liver disease beyond the typical history of cirrhosis after the acute infective period.



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Figure 2 Course of liver cirrhosis during COVID-19. UGI: Upper gastrointestinal; HRS: Hepatorenal syndrome; HE: Hepatic encephalopathy.

It is well known that infections put people at risk of decompensation (worsening ascites, encephalopathy, or acute kidney injury), and in the case of COVID-19, which is characterized by significant cytokine activation, cytokine-induced hepatocyte apoptosis and necrosis in the presence of decreased liver reserve may result in hepatic decompensation. To rule out COVID-19 as a possible cause, patients with cirrhosis who exhibit decompensation should be evaluated.

CLINICAL OUTCOMES OF PATIENTS WITH INDIVIDUAL UNDERLYING LIVER DISEASES

COVID-19 and Chronic HBV and HCV infection

As there are many etiologies (part of a systemic illness, immune mediated, direct SARS-CoV-2 infection, viral hepatitis, drug-induced, and ischemic hepatic injury) which can cause derangement of liver function tests, one of which is chronic HBV and HCV infection, it is always important to identify these underlying infections[59,60].

Prednisolone and tocilizumab have been used in the treatment of COVID-19, which are known to increase the likelihood of HBV reactivation and flare-up alongside HCV flare-up. When starting COVID-19-related therapy in those with advanced liver disease brought on by HBV and HCV, care must be taken[59,60]. Although the risk/benefit of an intervention is likely to weigh strongly when dealing with COVID-19, established criteria in such cases need to be followed to limit the risk of hepatic decompensation (Table 1).

COVID-19 and NAFLD

Risk factors in the general population for COVID-19 morbidity and mortality include advancing age, obesity, and diabetes[6]. With regard to how NAFLD affects the course of COVID-19, significant differences have been found in various studies. These differences may be attributable to problems in distinguishing the impact of NAFLD from other metabolic comorbidities due to the confounding effect of viral-induced steatosis or due to different diagnostic criteria. The latter point is especially crucial as the hepatology community at large struggles with the proposed classification modifications from NAFLD to metabolic dysfunction-associated liver disease[39]. Studies have shown that obesity is associated with increased severity and mortality in COVID-19. On the other hand, obese patients have a higher prevalence of diabetes, NAFLD, dyslipidemia, hypertension and metabolic syndrome. In a retrospective series of 202 patients with SARS-CoV2 infection, NAFLD was identified as a risk factor for progressive COVID-19, abnormal liver enzyme levels, and extended viral shedding times[61]. A study of 327 participants revealed an association between NAFLD and the likelihood of severe COVID-19 in people under 60 years of age[62]. Similar to this, MRI results from 287 SARS-CoV2 patients (79 positive, 208 negative) showed that obese patients with a concurrent liver fat fraction of less than 10% were three times more likely to develop symptoms of laboratory-confirmed COVID-19 (available as a non-peer-reviewed Preprint only)[63].

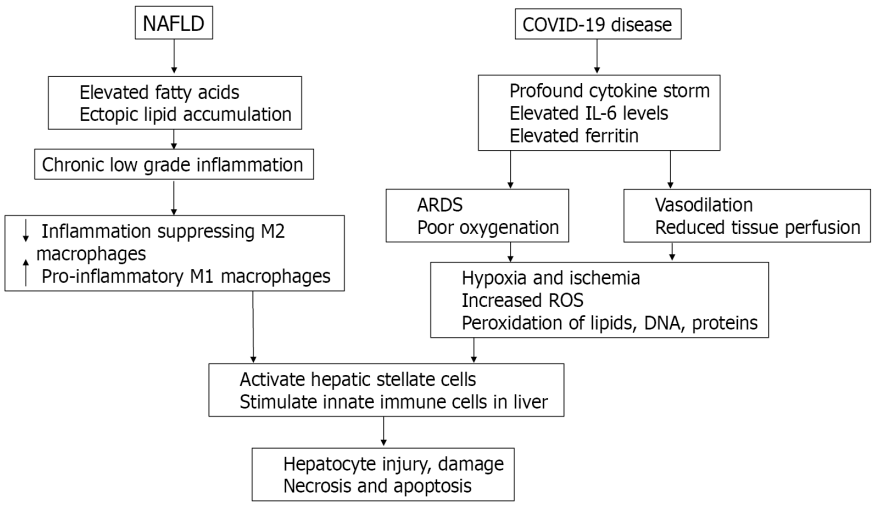
Table 1 Studies showing the effect of various etiologies of liver disease on COVID-19

Ref.	Country	Study design	Study population	Sample size	Outcome
HBV					
Anugwom *et al* [70], 2021	China	Letter	Peer reviewed articles with confirmed COVID-19 and HBV information	2054; HBV (*n* = 28)	Inverse relation of HBV with COVID-19
Kang *et al*[71], 2021	Korea	Retrospective, nationwide case-control study	Korean National Health Insurance Service COVID database	7723; HBV (*n* = 267)	Underlying chronic hepatitis B with COVID-19 severity adjusted odds ratio 0.65; 95%CI: 0.57-0.74)
HCV					
Richardson *et al*[15], 2020	United States	Case series	With confirmed COVID-19 and information on HCV infection	5700	HCV infections in < 0.1% (*n* = 3) of COVID-19 patients
Ronderos *et al* [72], 2021	United States	Retrospective single-center	With confirmed COVID-19 and information on HCV infection	1193; HCV (*n* = 50)	HCV infection predictor of in hospital mortality
NAFLD					
Ji *et al*[62], 2020	China	Retrospective	With confirmed COVID-19 and information on NAFLD status	202; NAFLD (*n* = 76)	HSI with disease progression (OR 6.4; 95%CI: 1.5-31.2)
Targher *et al* [64], 2020	China	Prospective observational	Laboratory confirmed COVID-19	310; NAFLD (*n* = 94)	FIB-4 (adjusted OR 1.90, 95%CI: 1.33 to 2.72) or NFS (adjusted OR 2.57, 95%CI: 1.73 to 3.82) with COVID-19 severity
Lopez-Mendez *et al*[65], 2021	Mexico	Retrospective	Medical records of hospitalized COVID-19	155; liver fibrosis (*n* = 69)	FIB-4 with risk of ICU admission (OR 1.74, 95%CI: 1.74-2.68; *P* = 0.023); mortality (OR 6.45, 95%CI: 2.01-20.83, *P* = 0.002)
Sachdeva *et al* [73], 2020	India	Systemic review	-	8142; NAFLD (*n* = 833)	Pooled adjusted 2.358 (95%CI: 1.902-2.923) with severity of COVID-19
Mahamid *et al* [74], 2021	Israel	Retrospective case-control	Medical records of COVID-19	71; NAFLD (*n* = 22)	OR 3.57 (95%CI: 1.22-14.48) with severity of disease
Hashemi *et al* [75], 2020	United States	Multicentre retrospective	Laboratory confirmed COVID-19	363; NAFLD (*n* = 55)	aOR 2.30 (95%CI: 1.27-4.17) with ICU admission
Yao *et al*[76], 2021	China	Retrospective	Laboratory confirmed COVID-19	86; NAFLD (*n* = 38)	OR 11.057 (95%CI: 1.193-102.439, *P* = 0.034) with severe COVID-19
Li *et al*[77], 2022	China and United States	Observational; 2-sample Mendelian randomization	Laboratory confirmed COVID-19	8267; NAFLD (*n* = 136)	OR 0.97 (95%CI: 0.88-1.08, *P* = 0.61) with COVID-19
BCS					
Espinoza *et al* [78], 2021	Brazil	Case report	Laboratory confirmed COVID-19	-	Thrombosis of an abdominal vessel should be considered as a differential diagnosis in patients with undefined abdominal pain and elevated liver biochemical tests
Sh Hassan *et al* [79], 2021	Saudi Arabia	Case report	Laboratory confirmed COVID-19	-	Thromboembolic events could be the first manifestation of COVID-19

COVID-19: Coronavirus disease 2019; HBV: Hepatitis B virus; HCV: Hepatitis C virus; BCS: Budd-Chiari syndrome; HSI: Hepatic steatosis index; FIB-4: Fibrosis-4; NAFLD: Non-alcoholic fatty liver disease; NFS: Non-alcoholic fatty liver disease fibrosis score; 95%CI: 95% confidence interval; OR: Odds ratio.

The chronic low-grade inflammation in NAFLD shifts macrophages from M2 to M1 phenotype and causes activation of hepatic stellate cells and the innate immune system which in collaboration with profound systemic inflammation in COVID-19 leads to hepatocyte injury, necrosis, and apoptosis (Figure 3).

Targher *et al*[64] reported high fibrosis-4 and NAFLD fibrosis scores with increased COVID-19 severity. Similarly, Lopez-Mendez *et al*[65] showed steatosis and fibrosis to be linked to increased ICU admissions. However, due to the constraints of isolation, quarantine and adequate manpower, there was a lack of detailed history and tissue histology; therefore, we do not have comparative studies of liver steatosis, steatohepatitis and fibrosis in relation to COVID-19 severity. The COVID-19 pandemic severely affected hepatology services in terms of early diagnosis, surveillance programs,



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Figure 3 Complex interplay of non-alcoholic fatty liver disease and COVID-19. COVID-19: Coronavirus disease 2019; NAFLD: Non-alcoholic fatty liver disease; ARDS: Acute respiratory distress syndrome; ROS: Reactive oxygen species; IL-6: Interleukin-6.

implementation of hepatitis B and C eradication programs, *etc* (Table 2).

COVID-19 and autoimmune hepatitis

Very little is known regarding the results of COVID-19 in individuals with autoimmune hepatitis (AIH), a rare form of CLD. The study by Marjot *et al*[66] in October 2020, included more than 1700 participants, and aimed to describe the course of COVID-19 and risk of unfavorable outcomes in 70 individuals with AIH. It was shown that despite the potential reporting of individuals with more severe liver disease, AIH does not significantly increase susceptibility to negative outcomes following SARS-CoV2 infection after several comparisons of non-AIH CLD and non-CLD cohorts. In contrast to the use of immunosuppressive agents, for which no adverse effects were found, age and the severity of baseline liver disease continue to be the most significant drivers of outcome in this patient group[45]. This should reassure patients and medical professionals, and support suggestions that immunosuppressive agents should not be frequently changed or stopped during COVID-19.

COVID-19 cholangiopathy

There are few case reports of secondary sclerosing cholangitis in patients with severe COVID-19 and histologic changes due to cholangiocyte injury and cholangiopathy. These patients had a protracted course and significant liver-related morbidity. Essentially, this condition was noted after recovery of COVID-19; therefore, it was called post COVID-19 cholangiopathy[67].

COVID-19 VIRAL ANTIGEN PERSISTENCE IN THE GUT

Recently, long-term sequelae of COVID-19 have been identified with symptoms of fatigue, insomnia, body ache and cognitive dysfunction. Persistence of viral antigens in gut epithelia have been documented[68]. It is possible that these persistent antigens cause immune dysfunction and low-grade persistent inflammation which manifests in various ways. It could be a basis for immune perturbation in post COVID-19. Its effect on liver in the post COVID era will be an area for research.

ADVERSE EFFECT OF mRNA VACCINES

The effects of mRNA vaccines for COVID-19 prevention have been implicated in the causation of "immune mediated hepatitis" due to the production of antibodies against the spike protein of SARS-CoV2 virus[69]. It will be interesting in the near future to detect autoimmune hepatitis or immune mediated hepatitis prevalence in the community.

Table 2 Impact of COVID-19 pandemic on hepatology services

Decrease	Increase
OPD follow-up and care	Inhospital admission
HBV treatment	Alcohol intake
HCV community level programs	HCC incidence
HCC surveillance and screening	Acute on chronic liver failure
UGI endoscopy	Gastrointestinal bleeding especially variceal bleeding
Liver transplantation	Unhealthy lifestyle
	NAFLD/MAFLD

OPD: Outpatient; COVID-19: Coronavirus disease 2019; HCC: Hepatocellular carcinoma; UGI: Upper gastrointestinal; NAFLD: Non-alcoholic fatty liver disease; MAFLD: Metabolic associated fatty liver disease.

CONCLUSION

During COVID-19, liver enzymes may be mildly elevated and generally recover without treatment. The presence of NAFLD has been linked to increased COVID-19 severity and ICU admissions. Different studies have shown the variable impact of NAFLD on COVID-19 related mortality. In patients with chronic hepatitis B and hepatitis C, a mild COVID-19 course is well tolerated, whereas in moderate-severe COVID-19 requiring steroids and/or tocilizumab, the risk of viral flare and worsening of liver disease is present. Patients with compensated cirrhosis are at increased risk of decompensation after COVID-19. In decompensated cirrhosis, the trajectory of COVID-19 severity and mortality rises with worsening Child-Pugh scores. With emerging evidence of persistent gut viral antigens capable of stimulating the immune system, we should be vigilant for postacute COVID-19 syndrome.

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FOOTNOTES

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Country/Territory of origin: India

ORCID number: Tarana Gupta 0000-0003-3453-2040; Hemant Sharma 0000-0001-8813-8933.

Corresponding Author's Membership in Professional Societies: American Association for the Study of Liver Diseases, No. 226223; Indian National Association for the Study of Liver Diseases, No. 1310.

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MINIREVIEWS

Nanomedicine-based multimodal therapies: Recent progress and perspectives in colon cancer

Yu-Chu He, Zi-Ning Hao, Zhuo Li, Da-Wei Gao

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Yu-Chu He, Zi-Ning Hao, Zhuo Li, Da-Wei Gao, State Key Laboratory of Metastable Materials Science and Technology, Nano-Biotechnology Key Laboratory of Hebei Province, Applying Chemistry Key Laboratory of Hebei Province, Yanshan University, Qinhuangdao 066000, Hebei Province, China

Corresponding author: Da-Wei Gao, PhD, Teacher, State Key Laboratory of Metastable Materials Science and Technology, Nano-Biotechnology Key Laboratory of Hebei Province, Applying Chemistry Key Laboratory of Hebei Province, Yanshan University, No. 438 Hebei Street, Qinhuangdao 066000, Hebei Province, China. dwgao@ysu.edu.cn

Abstract

Colon cancer has attracted much attention due to its annually increasing incidence. Conventional chemotherapeutic drugs are unsatisfactory in clinical application because of their lack of targeting and severe toxic side effects. In the past decade, nanomedicines with multimodal therapeutic strategies have shown potential for colon cancer because of their enhanced permeability and retention, high accumulation at tumor sites, co-loading with different drugs, and combination of various therapies. This review summarizes the advances in research on various nanomedicine-based therapeutic strategies including chemotherapy, radiotherapy, phototherapy (photothermal therapy and photodynamic therapy), chemodynamic therapy, gas therapy, and immunotherapy. Additionally, the therapeutic mechanisms, limitations, improvements, and future of the above therapies are discussed.

Key Words: Colon cancer; Nanomedicine; Drug permeability; Drug retention; Multimodal therapies; Therapeutic mechanism

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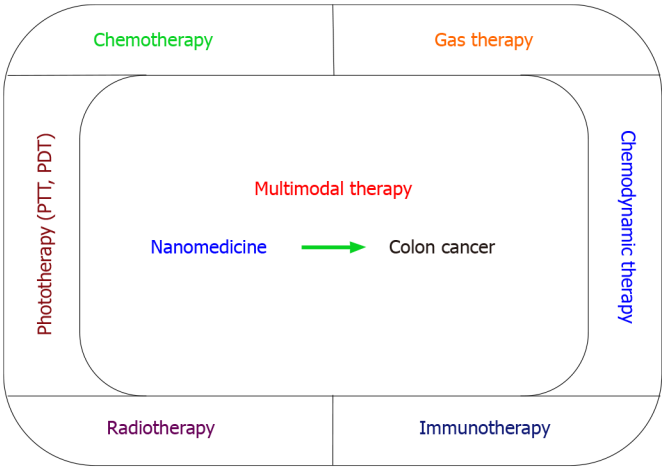
Core Tip: Nanomedicine has exhibited great potential in the colon cancer therapy over the past decades. In this review, we summarize the advances in research on various nanomedicine-based therapeutic strategies including chemotherapy, radiotherapy, phototherapy (photothermal therapy and photodynamic therapy), chemodynamic therapy, gas therapy, and immunotherapy. Additionally, the therapeutic mechanism, limitations, and improvement in these therapies are also introduced. The challenges and future prospect of the nanomedicine-based multimodal therapies for colon cancer are discussed.

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INTRODUCTION

Colon cancer is one of the most intractable gastrointestinal diseases with increasing incidence worldwide[1,2]. For the past few years, human lifestyles and diets have changed markedly with the rapid development of the global economy, which further increases the risk of colon cancer. According to the global cancer statistics, the incidence and mortality of colon cancer were 6.1% and 5.8% in 2018[3], which ranked fourth and fifth among all cancers, respectively. The characteristics of colon cancer are mainly reflected in rapid energy metabolism and proliferation that enhance tumor invasion and metastasis. Therefore, colon cancer has become one of the major unresolved problems in medicine[4,5]. Conventional small molecule chemotherapeutic drugs (such as paclitaxel, doxorubicin, and camptothecin) are unsatisfactory because of their lack of targeting and solubility, and severe toxic side effects. Thus, there is an urgent need to develop novel and efficient therapeutic strategies for colon cancer. In the past decade, the emergence of nanomedicine has shown potential in cancer therapy. Compared with traditional chemotherapeutic drugs, nanomedicine has better tumor targeting because the vascular gaps in tumor tissue are wider than those of normal tissue, so that nanomedicine can penetrate tumor tissue through these vascular gaps but not into normal tissue. Because of the lack of lymphatic reflux in the tumor region, nanomedicine can remain in the tumor tissue, and this mechanism of nanomedicine-based tumor targeting is called the enhanced permeability and retention (EPR) effect [6]. Additionally, various nanoscale drug delivery systems can load the chemotherapeutic drugs to enhance their solubility, which improves their utilization. Finally, nanomedicine is able to combine multimodal therapies to enhance the antitumor effect. Above all, nanomedicine has shown numerous advantages and potential for multimodal therapy of colon cancer.

In this review, we summarize recent progress of nanomedicine-based multimodal colon cancer therapy. First, we introduce all types of organic and inorganic nanomedicine and explore their drug loading, drug release, and tumor targeting. Moreover, the biosafety of nanomedicine is also discussed. Then, we introduce various therapeutic strategies for colon cancer including chemotherapy, phototherapy [photothermal therapy (PTT) and photodynamic therapy (PDT)], radiotherapy, gas therapy, chemodynamic therapy (CDT), and immunotherapy (Figure 1). The therapeutic mechanisms of these approaches are also discussed. Among them, nano drug delivery systems (NDDSs) are widely used to improve the therapeutic effect due to their characteristics of improving the water solubility of chemotherapy drugs, prolonging the blood circulation time, targeted drug delivery, few side effects, and reversing multi-drug resistance. PDT is a new treatment for colon cancer that uses specific wavelengths of light to excite photosensitizers. In the excited state, the photosensitizers transfer energy or electrons to the surrounding oxygen, thus producing singlet oxygen and killing cancer cells. Radiation therapy can cause DNA strand break of tumor cells under X-ray irradiation, and produce high cytotoxic free radicals to damage colon tumor cells. Compared with other reactive oxygen species (ROS) therapies, CDT has stronger *in situ* catalytic ROS generation, higher tumor specificity, and deeper tissue penetration, and does not require additional stimulation, providing a new idea for the future treatment of colon cancer. Gas therapy can enhance drug release, and when used with chemotherapy and synergistic therapy with other therapies, it can improve therapeutic effects, but its application in colon cancer requires extensive studies. Immunotherapy has been widely used in the treatment of colon cancer. The immunogenicity of tumor cells is activated by means of photothermal and ROS, and immunoadjuvant is used to reduce the immunosuppression in the tumor microenvironment and enhance the immune effect. These strategies provide new insights into the clinical treatment of colon cancer. Finally, the main limitations and challenges in the development of nanomedicine for colon cancer are addressed, and future research directions proposed. It is believed that nanomedicine-based multimodal therapy will play an important role in colon cancer.



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Figure 1 Schematic illustration of nanomedicine-based multimodal therapies for colon cancer. PTT: Photothermal therapy; PDT: Photodynamic therapy.

MULTIMODAL THERAPIES FOR COLON CANCER

Chemotherapy

Chemotherapy is the core method in current cancer treatment, and various drugs such as 5-fluorouracil (5-FU), platinum drugs, irinotecan, and epirubicin, are widely used[7-11]. However, there are still some problems in conventional chemotherapy: (1) Free small-molecule drugs have a limited half-life *in vivo* and lack of tumor targeting, leading to severe side effect; (2) Poor aqueous solubility of drugs limits their clinical effect; (3) Dense solid tumor tissue hinders drug delivery, resulting in insufficient drug dose in tumor tissue; and (4) Tumor microenvironment, such as hypoxia, low pH, and high H_2O_2 concentration, leads to multidrug resistance. To improve the therapeutic effect of chemotherapy, NDDSs have received extensive attention because of their properties such as improving the aqueous solubility of drugs, prolonging the blood circulation time, achieving targeted delivery to tumors, and few side effects. Various NDDSs have been designed to enhance tumor targeting and aqueous solubility of drugs, leading to improved therapeutic effect[12-15].

Most drugs exhibit poor aqueous solubility and low bioavailability. To solve this problem, Chen *et al*[16] adopted a cucurbituril-based supramolecular chemical strategy to improve the aqueous solubility and long-term circulation of the drugs for enhancing the therapeutic effect of oxaliplatin on colon cancer. Chen *et al*[17] prepared fisetin micelles using monomethyl poly(ethylene glycol)-poly(ε-caprolactone) copolymers. Compared with free fisetin, the micelles exhibited excellent aqueous solubility and cytotoxicity. Additionally, Xiao *et al*[18] used the intermolecular noncovalent interaction of curcumin and irinotecan to self-assemble into nanoparticles, which enhanced the aqueous solubility of curcumin, reduced the side effects of irinotecan, and showed better targeting and therapeutic effect. To prolong the blood circulation of drugs, Jiang *et al*[19] designed OxPt/SN38 nanoparticles to hitchhike on low-density lipoprotein (LDL) particles and accumulate at the tumor site through LDL-receptor-mediated endocytosis, which showed excellent antitumor efficacy in murine tumor models. Liu *et al*[20] developed an active targeting strategy to specifically combine glucose-regulated protein 78 overexpressed on the surface of colon cancer cells with PEGylated WL8 peptide, which enhanced the enrichment of doxorubicin in the tumor region.

Inflammation is an important reason for promoting tumor proliferation, invasion, metastasis, and drug resistance. Therefore, anti-inflammatory drugs such as aspirin and dexamethasone can improve the therapeutic effect of antitumor drugs[21,22]. Natural products such as curcumin and fisetin, which show good anti-inflammatory and antitumor properties, have also been widely used as chemotherapeutic drugs[23-26]. Wang *et al*[27] found that the anti-inflammatory drug dexamethasone significantly enhanced the antitumor activity of carboplatin and gemcitabine and increased their accumulation in tumors, providing a basis for dexamethasone as a chemosensitizer. Ma *et al*[28] developed a pH- and redox-responsive peptide-dexamethasone conjugate (L-SS-DEX) that reduces inflammation and modulates the tumor microenvironment for an effective antitumor effect.

Multidrug resistance is another reason for the failure of chemotherapy. The multidrug-resistance-related proteins such as P-glycoprotein (P-gp) of tumor cells result in significant drug excretion[29,30]. Currently, some NDDSs have been designed to co-deliver P-gp inhibitors or microRNAs to suppress multidrug resistance and enhance the drug sensitivity of tumor cells[31,32]. Sivak *et al*[33] overcame multidrug resistance by simultaneously delivering doxorubicin and the P-gp inhibitor (reversin 121) into cancer cells. The neurokinin-1 receptor antagonists inhibited expression of P-gp to enhance the

chemotherapy effect[34].

Studies have shown that the development of colon cancer is closely related to the gut microbiota, which is involved in regulating the sensitivity of tumor cells to chemotherapy. As a Gram-negative anaerobic bacterium, *Fusobacterium nucleatum* (*F. nucleatum*) is enriched in colon cancer patients, adheres to the intestinal mucosa, and invades epithelial cells to induce carcinogenesis. It can combine with E-cadherin on the surface of colon cancer cells to form a tumor immunosuppressive microenvironment, promote tumor proliferation, and enhance drug resistance of colon cancer cells[35-38]. Therefore, inhibiting the activity of *F. nucleatum* is important for enhancing the efficacy of colon cancer chemotherapy. Lauric acid has a specific inhibitory effect on *F. nucleatum*. Yan *et al*[39] used polyglycidyl ether as a nanodrug carrier, introduced the antibacterial agent lauric acid and oxaliplatin through esterification, selectively inhibited the biological activity of *F. nucleatum*, and improved the resistance of colon cancer cells to oxaliplatin. The antibiotic metronidazole and the chemotherapy drug 5-FU were mixed into the metal polyphenol network coated mesoporous silica nanoparticles (MSNs), and then added with carboxymethyl cellulose to obtain anti-colorectal cancer gel to eliminate *F. nucleatum* in colon cancer and inhibit the drug resistance, and proliferation and metastasis of colon cancer cells[40].

Phototherapy

Phototherapy is an emerging strategy to kill tumor cells by stimulating photosensitizers under light irradiation. In recent years, phototherapy, as a noninvasive treatment, has attracted widespread attention because of its specificity, low toxicity for normal tissues, and excellent antitumor effect. PTT and PDT are two common methods in colon cancer treatment[41-44]. PTT utilizes photosensitizer accumulated in tumor tissue to convert light energy into heat for killing tumor cells under light irradiation (generally near-infrared, NIR), which shows spatiotemporal controllability, high selectivity, and low cost. Recently, NDDSs have been designed to delivery photothermal agents for enhancing tumor targeting. For example, Ren *et al*[45] designed CT26 cell membrane-coated Bi nanoparticles, which had good long-term circulation and tumor homologous targeting ability *in vivo* compared with Bi nanoparticles. In addition, it is reported that epidermal growth factor receptor (EGFR) is abundantly expressed on the surface of some colorectal cancer cells. Shih *et al*[46] combined cetuximab (EGFR inhibitor) with the organic NIR dye IR780 to target colon cancer cells with high EGFR expression for PTT. Excessive H_2S (0.3-3.4 mmol/L) produced by colon cancer cells can promote the proliferation of colon cancer cells and angiogenesis in the tumor area[47,48]. Biocompatible iron oxide nanospindles have been developed, which can efficiently remove endogenous H_2S gas in colon tumor tissues and inhibit tumor growth, and generate FeS *in situ* for magnetic resonance imaging (MRI) and PTT under NIR irradiation[49-51].

PDT is a new method for colon cancer therapy that utilizes light of a specific wavelength to excite a photosensitizer, and the photosensitizer in the excited state transfers energy or electrons to the surrounding oxygen, thereby producing singlet oxygen to kill cancer cells[52]. Various NDDSs have been designed to deliver PDT-based photosensitizers to colon tumors. By adjusting the size of the NDDSs and modifying with hydrophilic groups, the photosensitizers can be passively targeted to the tumor area through the EPR effect. Besides the EPR effect, biomimetic membrane or tumor-specific affinity ligands-modified NDDSs have also been extensively studied for tumor targeting. Xie *et al*[53] designed a translocator protein (TSPO)-targeted photosensitizer (IR700DX-6T) for tumor targeting of photosensitizers *via* combination with overexpressed TSPO in colon cancer cells. Additionally, because of the high expression of EGFR in colon cancer cells, EGFR antibody has been used to target delivery of the photosensitizer IR700, which effectively eradicated colon cancer cells[54]. Traditional photosensitizers have high fluorescence quantum yields in dilute solutions, which leads to weaker fluorescence in the aggregated state. Aggregation of photosensitizers during delivery can lead to reduced ROS yields, so it is crucial to develop novel nanocarriers that efficiently load photosensitizers and prevent their aggregation. Covalent organic frameworks as a class of organic polymers, have attracted much attention because of their excellent biocompatibility and biodegradability. Gan *et al*[55] showed enhanced phototherapeutic effects by adsorbing the NIR dye indocyanine green (ICG) onto the covalent organic framework *via* π–π interaction to prevent its aggregation. In addition to this, aggregation-induced emission luminescence agents have been used to enhance PDT because the agents exhibit enhanced fluorescence emission in the aggregated state[56]. Hypoxia is one of the main reasons for the poor effect of PDT. Thus, researchers have developed a variety of oxygen generators such as hemoglobin, MnO_2, and perfluorocarbon, to increase oxygen in the tumor to enhance the effect of PDT [57-59]. For example, He *et al*[60] designed gold nanocages coated with MnO_2 and hyaluronic acid (HA) for tumor targeting, and MnO_2 was designed to react with the overproduced H_2O_2 in the tumor to relieve tumor hypoxia and enhance the effect of gold nanocage-based PDT.

Radiotherapy

Radiotherapy is a local cancer treatment that is widely applied in clinical therapy. The mechanism of action of radiotherapy is to cause DNA strand breaks in tumor cells and generate highly cytotoxic free radicals under X-ray irradiation to damage tumor cells[61-65]. Radiosensitizers are usually used to boost the effect of radiotherapy against colon cancer[66]. 7-Dehydrocholesterol is utilized as a radiosensitizer,

which can react with ROS to promote lipid peroxidation, double-strand breaks, and mitochondrial damage in cancer cells, enhancing the radiotherapeutic effect[67]. As we know from the mechanism of action of radiotherapy, tumor hypoxia limits the efficacy of radiotherapy; thus, relief of hypoxia by nanomedicine can improve the therapeutic effect. MnO_2 can react with excess H_2O_2 in the tumor to generate oxygen, which can relieve the hypoxic microenvironment, eliminate tumor resistance to radiotherapy, and reshape the immunosuppressive microenvironment. Zhang *et al*[68] designed bovine-serum-albumin-coated MnO_2 as a radiosensitizer. MnO_2 can decompose excess H_2O_2 in the tumor into oxygen to relieve tumor hypoxia and convert tumor-promoting M2 tumor-associated macrophages into antitumor M1-type macrophages to reshape the immunosuppressive microenvironment and eliminate tumor resistance to radiotherapy. In addition, perfluorocarbon is a good oxygen carrier that can be used to delivery oxygen to tumors and reverse hypoxia, leading to enhancement of radiotherapy[69].

CDT

CDT is a promising therapeutic strategy that utilizes endogenously overexpressed H_2O_2 in tumors to generate toxic hydroxyl radicals ($\cdot OH$) through Fenton/Fenton-like reactions catalyzed by metals (Fe^{2+}, Cu^{+}, Mn^{2+}, Mo^{4+}, W^{4+}, Ti^{3+}, *etc.*)[70-73]. Compared with other ROS therapies, CDT has the advantages of stronger *in situ* catalytic ROS generation, tumor specificity, and deep tissue penetration, which does not require additional stimulation. However, the effect of CDT is still limited by its high dependence on tumor endogenous H_2O_2 concentration (10-100 μM) and slow ion release from inorganic nanoparticles [74,75]. The problem of low levels of H_2O_2 in tumor tissue can be solved by directly loading H_2O_2 or encapsulating H_2O_2-producing drugs such as glucose oxidase and calcium peroxide. However, nanocarriers directly encapsulating exogenous H_2O_2 have the risk of leakage causing damage to normal tissues. Therefore, new strategies are urgently needed to address the challenges associated with CDT. Su *et al*[76] used a microfluidic method to prepare a nanogel (DOX@Mn-Alg) composed of alginate (Alg), Mn^{2+}, and doxorubicin as an ideal CDT/chemotherapy synergistic therapeutic nanoplatform, because doxorubicin can activate NADP oxidases to convert oxygen to $\cdot O_2^-$ and then superoxide dismutase further catalyzes $\cdot O_2^-$ to generate endogenous H_2O_2 *via* a disproportionation reaction. Subsequently, the elevated H_2O_2 can be converted into a sufficient amount of $\cdot OH$ through a Mn^{2+}-mediated Fenton-like reaction. Ultimately, DOX@Mn-Alg can rationally combine doxorubicin chemotherapy with Mn^{2+}-mediated CDT and immunotherapy for synergistic cancer treatment. Chen *et al*[77] selected Pd nanoparticles as a CDT reagent, and showed that the ultra-small Pd nanozyme as the core had high catalytic activity and pH selectivity. Under acidic conditions, it exhibited peroxidase activity to produce OH and $^1O_2$, while under neutral conditions, it promoted the decomposition of H_2O_2 to produce O_2 through catalase activity. In terms of biological activity, the bidirectional anisotropic nanocluster not only directly inhibited tumor cells through ROS production, but also induced H_2O_2 production in CT26 cells, which enhanced the therapeutic effect. The nanoparticles inhibited tumor growth in CT26 mice, and improved tumor hypoxia and enhanced the therapeutic effect.

The intracellular glutathione in tumor cells can eliminate the oxidative activity of $\cdot OH$ through powerful reducing activity. Lin *et al*[78] devised a strategy to enhance CDT by inhibiting expression of glutathione in tumors and remodeling the reductive state of the tumor microenvironment, indicating that inhibition of glutathione can improve the effect of CDT. Wang *et al*[79] reported a degradable $MnSiO_3$ nanosystem for CDT/chemical synergistic therapy. First, $MnSiO_3$ nanoparticles were synthesized, and then the surface-initiated living radical polymerization of monomer of SN38 and oligo(ethylene glycol) methacrylate was conducted to obtain the product of CAMNSN@PSN38. Nanoparticles delivered to tumor tissues were gradually biodegraded by glutathione over time, during which SN38 and Mn^{2+} were gradually released. The released SN38 showed a favorable chemotherapeutic effect and increased accumulation of H_2O_2. The interaction of CAMNSN@PSN38 with glutathione depleted glutathione in tumor tissues and led to Mn^{2+} release for CDT and MRI-guided therapy. CAMNSN@PSN38 had a good inhibitory effect on colon tumor growth and assisted MRI-guided imaging through ROS accumulation *in vivo*. Unlike other tumor types, colon tumor shows high expression of H_2S (0.3-3.4 mmol/L), whose reductive activity is stronger than that of glutathione[80,81]. Therefore, in the treatment of colon cancer, the effect of CDT is also limited by endogenous H_2S. Liu *et al* [82] constructed $CuFe_2O_4$ nanoparticles to explore the potential of endogenous H_2S depletion to enhance CDT for colon cancer. $CuFe_2O_4$ nanoparticles remodel endogenous H_2S in colon cancer and enhance the Fenton or Fenton-like reaction of Cu(I) and Fe(II) by a photothermal effect to generate more $\cdot OH$. The results suggest that $CuFe_2O_4$ nanoparticles effectively enhance the effect of CDT by depleting H_2S. In addition, H_2S-responsive therapeutic nanoplatforms have been designed. Xiao *et al*[18] synthesized a copper-based metal-organic framework named HKUST-1 as a smart therapeutic platform. PTT and CDT were activated in the presence of H_2S in colon cancer cells. H_2S-triggered nanosystems can minimize side effects on surrounding normal tissues and precisely inhibit colon cancer growth. Above all, CDT shows potential for colon cancer treatment.

Gas therapy

As an emerging treatment method, gas therapy has attracted research interest in recent years[83-86]. Gas therapy refers to use of H_2S[87], NO[88], CO, *etc.* to kill tumor cells[89]. Liu *et al*[90] designed a nanoplatform (PEG/SCNPs@DMSN-SNO-g-C_3N_4) to release NO under X-ray irradiation, and then NO

reacted with superoxide anions to generate $ONOO^-$ toxic free radicals, leading to apoptosis through mitochondrial damage. NO has been proven to activate innate and adaptive responses of the immune system against tumors. Previous *in vivo* results showed that all NO-treated colon tumor-bearing (CT26 model) mice were resistant to secondary CT26 cell inoculation. Nonsteroidal anti-inflammatory drugs (NSAIDs) are prototypical anticancer agents. NO and H_2S are gaseous mediators with physiological relevance and NSAIDs that possess an H_2S- and NO-releasing moiety have shown beneficial effects. Chattopadhyay *et al*[91] synthesized and characterized a new class of anti-inflammatory NO- and H_2S -releasing compounds. This induced apoptosis, inhibited cell proliferation, and reduced colon tumor growth in a mouse xenograft model. Zhang *et al*[92] designed gas-generating MSNs, which can load ammonium bicarbonate and doxorubicin in the pores, and ICG coated on a polydopamine layer and modified with RGD peptides on the outer surface [M(ABC)-DOX@PDA-ICG-PEG-RGD] for triggering drug release and targeted chemotherapeutic photothermal combination treatment. At high temperature and low pH, the encapsulated ammonium bicarbonate can effectively generate CO_2. The CO_2 can damage the polydopamine layer and accelerate the release of doxorubicin. The results proved the excellent antitumor effect of gas therapy and chemotherapy, as well as good biosafety. Therefore, the gas therapy showed potential for colon cancer therapy.

Immunotherapy

Immunotherapy exhibits potential against colon cancer because it relies on the autoimmune system to attack malignant tumors. Immunotherapy for colon cancer is mainly divided into the following categories: (1) Activation of tumor immunogenicity; (2) Relief of tumor microenvironment immunosuppression; (3) Design of antitumor neoantigen vaccines and novel immune adjuvants; and (4) Design of therapeutic strategies using macrophages as target cells. However, only a subset of cancer patients responds to current immunotherapies because of the low immunogenicity of tumor cells and the immunosuppressive tumor microenvironment. Therefore, new strategies are needed to activate tumor immunogenicity and relieve the immunosuppression of the tumor microenvironment to improve the effect of immunotherapy. Fan *et al*[93] reported pH-responsive core-shell nanoparticles (HCLO NPs) for co-delivery of oxaliplatin intermediate and cytosine-guanine-containing oligodeoxynucleotide (CpG) for colon cancer treatment, and the oxaliplatin intermediate intratumoral injection induced *in situ* antigen production *via* immunogenic cell death. Subsequently, CpG enhanced antigen presentation and promoted production of cytotoxic T lymphocytes (CTLs). The results indicated that the HCLO NPs enhanced the toxicity of oxaliplatin intermediate for CT26 cells and upregulated expression of calreticulin, which exhibited significant immunity and antitumor effect. Hu *et al*[94] integrated HA, pheophorbide A heterodimer, and NLG919 into a supramolecular nanocomposite, which generated ROS under NIR laser irradiation to kill tumor cells, stimulated antitumor immunogenicity, and enhanced intratumoral infiltration of CTLs. The immunosuppressive tumor microenvironment was reversed by NLG919-mediated inhibition of indoleamine 2,3-dioxygenase 1. The results showed that this strategy could effectively kill CT26 colon tumors. Ding *et al*[95] designed liposome-encapsulating phosphatidylinositol 3-kinase γ inhibitor IPI-549 and photosensitizer Ce6 for immunotherapy of colon cancer. When the liposomes were internalized into CT26 cells, ROS were generated under laser irradiation, causing immunogenic tumor cell death. IPI-549 transported by liposomes promoted apoptosis of myeloid-derived suppressor cells and reduced the immunosuppressive activity of $CD8^+$ T cells to inhibit growth of CT26 tumors. Checkpoint inhibitors, such as antibodies that block the programmed cell death protein 1/programmed cell death ligand 1 (PD-1/PD-L1) pathway, are among the most promising immunotherapies for metastatic cancer. However, the responses rates remain low. To solve this problem, Yu *et al*[96] developed nanoparticles with PD-L1 blocking ability, which integrated PTT, antitumor immunity, and PD-1/PD-L1 blockade to enhance antitumor efficacy. In the mouse CT26 bilateral tumor model, intravenously injected nanoparticles accumulated at the tumor site and mediated a strong photothermal effect, eliminated the primary tumor by inducing immunogenic cell death, and elicited strong antitumor immunity. Growth of untreated distant tumors was inhibited by the synergistic effect of systemic antitumor immune activation and PD-L1 blockade. This strategy provided a promising approach for the treatment of metastatic cancer.

The reported immunoadjuvants have many limitations, such as poor cellular uptake and biocompatibility, excessive particle size, single function, and unsatisfactory therapeutic effect. Ding *et al*[97] prepared mesoporous silica-coated upconversion nanoparticles (UCMSs) and used them as a novel immune adjuvant. UCMSs had significant loading of the photosensitizer merocyanine 540, chicken ovalbumin, and tumor cell fragments. The UCMSs exhibited the best synergistic immune enhancement under 980 nm NIR irradiation, with the strongest Th1 and Th2 immune responses, and the highest frequencies of $CD4^+$, $CD8^+$, and effector memory T cells. In addition, nanovaccine UCMSs inhibited tumor growth more effectively and improved survival of tumor-bearing mice compared with PDT or immunotherapy alone, indicating that UCMSs have higher immunotherapeutic efficacy and clinical potential. As a new tumor vaccine based on zymosan shell particles[98], GP-Neoantigen can stimulate the body to generate a strong antigen-specific $CD8^+$ T cell immune response and an immune response to a variety of neoantigen peptides, and thereby be used for effective tumor treatment. The vaccine induced strong specific $CD8^+$ T cell immune responses and humoral immune responses *in vivo*, which also showed strong tumor growth inhibitory activity in the CT26 colon cancer model. Binding to Toll-

like receptor agonists PolyI:C and CpG 2395 enhanced the antitumor effect and achieved complete tumor clearance. These results provide broad possibilities for further clinical promotion and personalized vaccine therapy.

M2 macrophages are polarized by stimulatory factors in the tumor microenvironment and promote tumor growth. They are involved in limiting T cell function, tumor angiogenesis, and tumor invasion and metastasis. Increasing the ratio of M1/M2 macrophages in the tumor microenvironment is a promising cancer immunotherapy strategy. An erythrocyte membrane nanoparticle encapsulating *Porphyromonas gingivalis* can modulate the ratio of M1/M2 macrophages for cancer immunotherapy[99], and such nanoparticles inhibited the growth of primary and secondary tumors of CT26 colon cancer under the action of laser and anti-PD-1. Immunotherapy based on nanomedicine has been widely used in cell and animal models, and has shown good anti-tumor efficacy. It is expected to become one of the most potential therapeutic means in cancer treatment.

CONCLUSION

Several advanced nanomedicine applications have been developed for colon cancer therapy, which overcome the poor tumor targeting and efficacy of conventional drugs. This review presents various organic- and inorganic-based nanomedicines applied in colon cancer therapy using CT26 cells as the tumor model. We have introduced the mechanism of nanomedicine-based therapeutic strategies including chemotherapy, phototherapy (PTT and PDT), radiotherapy, gas therapy, CDT, and immunotherapy. These multimodal therapeutic strategies based on nanomedicine against colon cancer have shown excellent antitumor effect and potential.

Although the nanomedicine-based multimodal therapies have shown a superior effect against colon cancer, several limitations need to be overcome in future development. The first limitation is the unsatisfactory tumor penetration of nanomedicine. Drug delivery *in vivo* includes circulation, accumulation, penetration, internalization, and release. Poor tumor penetration has become a long-standing problem for the development of nanomedicine, which leads to the survival of tumor stem cells in deep tumor sites. The reason is the serious hinders of dense extracellular matrix and elevated tumor interstitial pressure. Thus, there is an urgent need to develop novel strategies to enhance tumor penetration of nanomedicine. The second limitation is obstruction of various therapies by the tumor microenvironment. For example, tumor hypoxia limits oxygen-dependent therapy such as PDT and radiotherapy. Additionally, M2 tumor-associated macrophages construct the tumor immunosuppression environment, which limits the effect of immunotherapy. Not only that, the immune checkpoint protein on the tumor cell inhibits the recognition and combination of cytotoxic T cells. Therefore, reversing the adverse effects of the tumor microenvironment is the key to improving the therapeutic effect of nanomedicine. It is expected that nanomedicine-based multimodal therapeutic strategies will have potential for clinical translation into colon cancer therapy.

FOOTNOTES

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ORCID number: Da-Wei Gao 0000-0002-5030-7022.

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MINIREVIEWS

Gaseous metabolites as therapeutic targets in ulcerative colitis

Chu K Yao, Chen Sarbagili-Shabat

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Chu K Yao, Department of Gastroenterology, Monash University, Melbourne 3004, Australia

Chen Sarbagili-Shabat, Pediatric Gastroenterology Unit, PIBD Research Center, Wolfson Medical Center, Holon 5822012, Israel

Corresponding author: Chu K Yao, PhD, RN, Senior Research Fellow, Department of Gastroenterology, Monash University, Level 6, Alfred Centre, 99 Commercial Road, Melbourne 3004, Australia. chu.yao@monash.edu

Abstract

Diet therapies are currently under-utilised in optimising clinical outcomes for patients with active ulcerative colitis (UC). Furthermore, existing dietary therapies are framed by poorly defined mechanistic targets to warrant its success. There is good evidence to suggest that microbial production of gaseous metabolites, hydrogen sulfide (H_2S) and nitric oxide (NO) are implicated in the development of mucosal inflammation in UC. On a cellular level, exposure of the colonic epithelium to excessive concentrations of these gases are shown to promote functional defects described in UC. Hence, targeting bacterial production of these gases could provide an opportunity to formulate new dietary therapies in UC. Despite the paucity of evidence, there is epidemiological and clinical data to support the concept of reducing mucosal inflammation in UC *via* dietary strategies that reduce H_2S. Several dietary components, namely sulphur-containing amino acids and inorganic sulphur have been shown to be influential in enhancing colonic H_2S production. More recent data suggests increasing the supply of readily fermentable fibre as an effective strategy for H_2S reduction. Conversely, very little is known regarding how diet alters microbial production of NO. Hence, the current evidence suggest that a whole diet approach is needed. Finally, biomarkers for assessing changes in microbial gaseous metabolites in response to dietary interventions are very much required. In conclusion, this review identifies a great need for high quality randomised-controlled trials to demonstrate the efficacy of a sulphide-reducing dietary therapy for patients with active UC.

Key Words: Diet; Ulcerative colitis; Hydrogen sulfide; Nitric oxide; Sulphide-reducing diet

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Core Tip: There is room to develop efficacious dietary therapies in ulcerative colitis (UC) by targeting underlying pathogenic mechanisms. Emerging data indicates that dietary factors play a significant role in modulating two gaseous metabolites, hydrogen sulphide and nitric oxide, that affect the integrity of the colonic mucosal barrier in UC. These gases are produced by the colonic microbiota in response to sulphur-containing protein and to a lesser extent, inorganic sulphur (sulphates and sulphites), but suppressed by the presence of fermentable fibre. Preliminary work suggests that a multi-prong diet that targets reduction of these gases have therapeutic potential and further randomised-controlled trials are underway.

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INTRODUCTION

Ulcerative colitis (UC) is characterised by chronic inflammation of the colonic epithelium as a result of an aberrant immune response to poorly understood initiating triggers[1]. Diet is a well-recognised environmental factor in the development of UC[1,2], but remains an under-utilised therapeutic tool amongst physicians and dietitians alike. Dietary management is currently directed at providing supportive symptomatic management. However, in the recent years, there has been a dogma shift towards harvesting dietary therapies with mechanistic targets for the induction of disease remission, as evidenced by the growing number of review articles in the area[3-5].

Whilst most research have been focused on altered immune regulation in the early initiative events, there is now a good body of evidence generated over the last 20 years suggesting that UC is an epithelial disease[6]. Metabolic defects in the colonic epithelium are central in its pathogenesis and may be responsible for mucosal barrier breakdown[7]. In turn, microbial metabolites such as hydrogen sulfide (H_2S) and nitric oxide (NO) that are toxic at excessive concentrations, may further exert injurious effects on the epithelium[8]. Diet is a major factor in colonic production of these metabolites. Hence, dietary strategies that minimise their production mechanistically may have therapeutic benefits in UC. This review aims to examine the evidence for H_2S and NO as causative agents in UC, the influence of diet on their colonic metabolism and to explore the rationale as well as evidence to date for dietary strategies targeting these gaseous metabolites as a therapy in UC.

COLONIC PRODUCTION OF H_2S & NO

Luminal H_2S is derived solely from metabolic activities of the microbiota, namely from fermentation of sulfur-containing amino acids and dissimilatory sulfate reduction[9]. Approximately 6-18 g/d of proteinaceous substrates are delivered to the colon for fermentation, the bulk of this originating from undigested dietary protein and a smaller proportion from endogenous protein secretions[10]. A range of protein-fermenting microbes with the capacity to generate H_2S have been reported including *Escherichia coli, Clostridium spp., Bacteroides spp.* and *Klebsiella pneumonia*[10]. In contrast, the capacity to reduce sulfate within the microbiota appears to be limited. A smaller proportion of malabsorbed dietary inorganic sulfur (0.3-8 mmol/d)[11] reach the colon as substrates for dissimilatory sulfate reduction. Sulfate- and sulfite-reducing bacteria such as *Desulfovibrio spp.* and *Bilophila wadsworthia* are highly specialised microbes with capacity for sulfate reduction[11].

On the other hand, two major sources of luminal NO are known: (1) Mucosal production from arginine; or (2) Anaerobic bacterial denitrification which reduces nitrates to nitrites and to NO[8]. To date, little work has been done to examine microbial populations capable of denitrification. Hence, the understanding of microbial pathways for gaseous production has important implications not only as potential therapeutic targets but has significant relevance for manipulation of dietary substrates.

ROLE AS LUMINAL TOXINS IN PATHOGENESIS OF UC

The most compelling argument for the colonic epithelium as the primary defect in UC has been derived from *ex vivo* studies showing diffuse structural and functional abnormalities in the absence of histological or endoscopic inflammation[12-14]. A key functional defect identified is the impaired uptake and oxidation of butyrate by colonocytes for energy[15,16]. As a result, the energy-starved colonic epithelium has limited ability to perform other metabolic functions including the maintenance of

barrier function. Furthermore, reduced structural integrity of the colonic mucus layer was reported by van der Post *et al*[13]. This was characterised by a marked decrease in core mucus components in both inflamed and non-inflamed biopsy samples, with similar findings reported previously[14]. Hence, the induction of mucosal inflammation may occur as a secondary response to the increased intestinal permeability[6].

Several lines of observations support the involvement of luminal H_2S and NO in perpetuating functional defects of the colonic epithelium. These concepts are summarised in Figure 1. First, Levine *et al*[17] showed that faecal release of H_2S was three-fold higher and more rapid in UC patients (both active and quiescent) compared to controls. Additionally, a greater relative abundance and activity of sulfate-reducing microbes, *Desulfovibrio*, has been documented in faecal or mucosal biopsy samples of patients with UC compared to non-UC controls[18,19]. Gut dysbiosis may be the main pathogenic factor of UC, and the higher dominance of sulphate-reducing microbes may potentially contribute to the dysbiosis hypothesised in the pathogenesis of UC. No data currently exists of potential alterations to the abundance of protein-fermenting microbes in UC. Furthermore, in contrast to a healthy colonic epithelium where H_2S is effectively detoxified, enzymatic detoxification activity of H_2S have been shown to be significantly depressed in UC[20]. Finally, elevated luminal H_2S concentrations are shown to be directly proportional to the severity of disease[16,17], providing an evidence base for a pathogenic link with UC. Likewise, direct assessment of luminal NO using a rectal balloon in patients with active UC demonstrated markedly higher rectal NO levels in these patients compared to those with irritable bowel syndrome and healthy controls[21].

Secondly, reduced carbohydrate fermentative ability, as was recently reported[22], and decreased accessibility to short-chain fatty acids[23] may have lead-on effects on altered sulfur metabolism. Insights gained by assessment of intestinal pH responses to dietary manipulation of fermentable fibres suggest that abnormalities in carbohydrate fermentative ability may be region specific[24]. Reduced butyrate utilisation may increase luminal accumulation of H_2S as its regulatory role on detoxification pathways are affected[25]. On the other hand, fibre deprivation may act synergistically with H_2S to increase breakdown of the mucous layer[26].

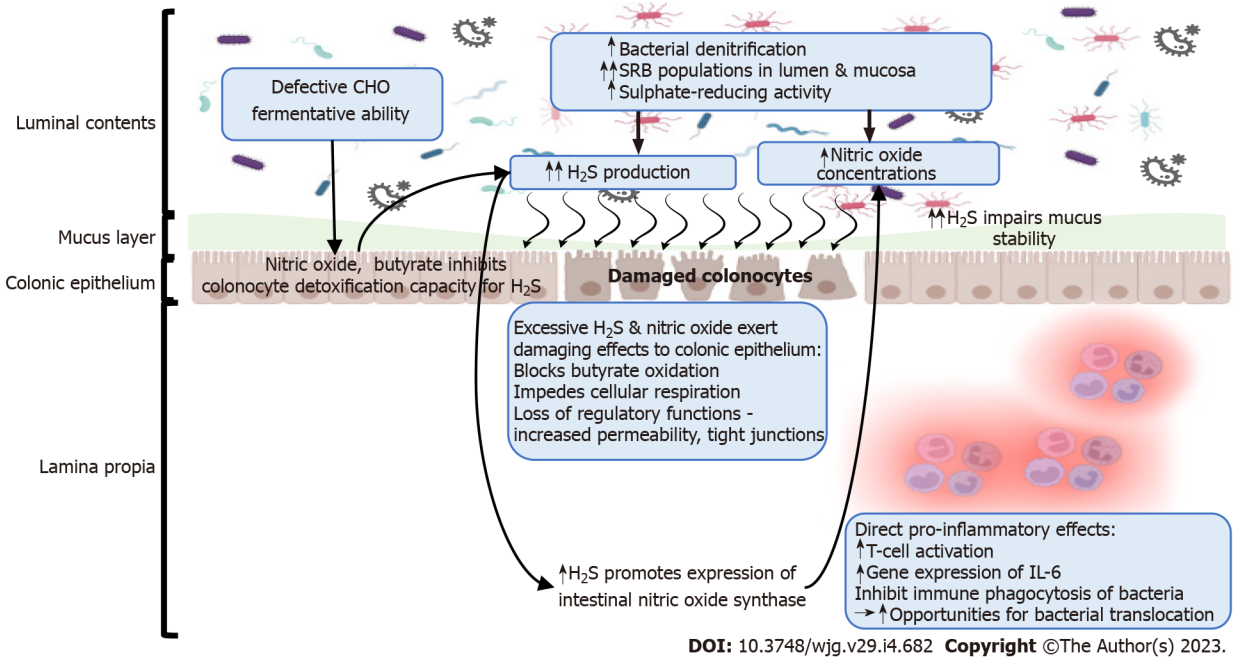
Thirdly, at excessive concentrations, continuous exposure of isolated colonocytes to combined H_2S and NO *in vitro* can produce extensive disruption of the epithelial barrier by interfering with cell membrane synthesis[8], impeding butyrate oxidation and subsequently, cellular respiration, producing an energy-deficient state as described earlier. This theory was confirmed by Leung *et al*[27] who induced a histological state that was similar to the pathology of UC in the colon of rats administered with sulfates (carrageenan). Furthermore, excessive H_2S and NO may exert other pathogenic effects, including direct immune effects and these are summarised in Figure 1.

Hence, restricting epithelial exposure to luminal H_2S and NO *via* reduced microbial production may hypothetically improve epithelial function and reduce mucosal inflammation in UC, a novel therapeutic strategy that was proposed two decades ago[28] but has only achieved some progress in the last two years. Progress is hampered by difficulties in accurate measurements of luminal H_2S and NO to provide a biomarker for assessing the efficacy of interventions on these metabolites. These challenges are discussed further in the subsequent sections.

DIET AS PRIMARY STRATEGY FOR COLONIC H_2S & NO MANIPULATION

From discussions above, it can be hypothesised that a key strategy in reducing microbial H_2S and NO production is by reducing substrate availability. Food choice represents a rationale candidate for manipulation as substrate delivery to the colon is strongly influenced by dietary intake. Indeed, several lines of evidence exist supporting the efficacy of dietary manipulation on colonic H_2S production. In contrast, the influence of diet on the extent of bacterial denitrification has been inconsistently shown.

First, acute dietary studies in healthy controls changing from low to a high animal protein diet consistently raised faecal H_2S levels[29,30]. Magee *et al*[29] reported this increase in H_2S levels to be linear with increasing intake of red meat (from 0 to 600 g/d). In another study, a four-day animal-based diet specifically increased a sulfite-reducing species, *Bilophila wadsworthia*, while a plant-based diet reduced this cluster[31]. Similarly, the animal-based diet significantly increased sulfide reductases needed for H_2S production[31]. Another source of inorganic sulfur in the diet occurs naturally in the form of glucosinolates in the Brassica vegetables family. However, a two-week diet high in brassica was associated with a reduction in the abundance of sulphate-reducing bacteria in a randomized crossover study with ten healthy adults[32], which seems to indicate that natural inorganic sulfur is not a determining factor in H_2S production associated with sulfate-reducing bacteria. Thirdly, whilst assessment of sulfate-reducing bacteria may be useful, it does not provide a comprehensive picture of functional alterations in microbial H_2S metabolism *in vivo*. Preliminary insights were gained with the use of a gas-sensing technology incorporating real-time, accurate measurements of H_2S to enable further understanding of the extent of dietary influence on microbial H_2S production[33]. A comparison between faecal slurries spiked with cysteine, a sulfur-containing amino acid, and sodium sulfate showed marked differences in faecal H_2S generation, with cysteine vigorously stimulating H_2S over



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Figure 1 Proposed mechanisms of microbial metabolites, hydrogen sulfide and nitric oxide, in the pathogenesis of ulcerative colitis *via* a dysfunctional colonic epithelium and breakdown in mucosal barrier function. Figure summarised from references[8,55-57]. CHO: Carbohydrate; H_2S: Hydrogen sulfide; SRB: Sulfate-reducing bacteria; H_2S: Hydrogen sulfide; IL: Interleukin.

sulfate. This finding indicates that protein fermentation may be a major pathway for H_2S production than dissimilatory sulfate reduction. Furthermore, faecal H_2S was effectively reduced by readily fermentable fibres, resistant starch and fructo-oligosaccharides, both of which are prebiotics, and even in the presence of excessive faecal H_2S production using cysteine[33]. The likely mechanism for H_2S suppression by fermentable fibre in the presence of cysteine is the shift from protein to carbohydrate fermentation as microbes preferentially ferment fibre than protein[10]. Suppression of H_2S has been reported by another study where a 1.5-fold increase in total dietary fibre that accompanied the reduction in animal protein had a negative impact on H_2S production[30] and in a second study, the addition of resistant starch to a high meat diet reduced markers of protein fermentation including H_2S [34]. Both inulin and fructo-oligosaccharides, well-established prebiotics were also shown to reduce H2S levels in pigs[35].

In addition, one of the strategies targeting the microbiota is probiotics with specific probiotic strains shown to be effective in inducing remission in active UC[36]. However, its properties on the gut microbiota warrants further investigation, particularly with regards to the influence of different probiotic strains on H_2S production. On the other hand, a promising probiotic treatment for UC is recent development of a 'smart probiotic' where E. coli Nissle 1917 was genetically engineered to detect colonic NO and would theoretically be able to release biologic therapy at the site of elevated colonic NO[37]. This engineered probiotic had previously been shown to have positive impact on the intestinal barrier function, and were able to reduce inflammation in dextran sulfate sodium -induced colitis mice model [38]. Prebiotics are another key player in microbiome manipulation that have been suggested to have a positive effect on the microbiome. Their mechanisms in modulating microbial H_2S have already been discussed earlier. However, randomized controlled trials (RCTs) in UC patients that evaluated the efficacy of prebiotic supplementation alone demonstrated limited weak effect[39] which indicates that a multi-prong approach, not just prebiotic supplementation, is required to achieve clinical effects.

Secondly, there is some evidence from epidemiological studies that provide clues for the influence of dietary sulfur-containing protein, sulfates and sulfites on the clinical course of UC. One study reported a correlation between a high protein intake and increased risk of developing UC[40] whilst only one study has shown an association between a high intake of sulfur amino acids and sulfate with a three-fold greater risk of disease relapse[41]. Subsequently, the potential clinical efficacy of a sulfur-restricted diet was first described from a small open-label study in eight UC patients. The low sulfur diet combined with stable salazopyrin therapy was associated with histological and clinical improvement[9]. Changes in colonic H_2S production was unfortunately, not measured as a mechanism for efficacy but the promise of this dietary approach warrant further investigation in a controlled trial.

Furthermore, there has been growing interest in the role of carrageenan, a sulphated polysaccharide food additive that escapes digestion in the small intestine almost intact and is fermented to release sulphates[42], which is then metabolised to produce H_2S. In 2017, a RCT by Bhattacharyya *et al*[43] assessed the role of carrageenan, a sulphated polysaccharide food additive, in maintaining relapse in 14

UC patients in remission. Following a year of no-carrageenan diet, relapse rates appeared to be higher in the five patients receiving 200 mg carrageenan, a dose slightly below average intakes of carrageenan in the US diet, than those who received placebo capsules. Unfortunately, significant recruitment issues impacted on the sample size of the study, making it difficult to ascertain whether this was a real clinical effect.

Finally, the major sources of nitrites in the diet are food preservatives in cured and processed meat, while the major source of dietary nitrates is vegetables[44]. Thus far, the effect of different sources of nitrite and nitrate in the diet on microbial NO production are unknown and should be further investigated in clinical studies.

TRANSLATING PROPOSED DIETARY STRATEGY INTO CLINICAL APPLICATION

Several dietary strategies can therefore be implied for future clinical application from studies thus far. First, a multi-prong intervention targeting dietary substrates with H_2S-modulating abilities, expanding on Roediger[9]'s earlier work, is warranted in active UC patients. This approach should consider reducing intake of sulfur-containing protein such as methionine, cysteine and taurine, and added sources of inorganic sulfur to reduce excessive/chronic H_2S exposure to the colonic epithelium. Major sources of these foods are listed in Table 1. Inorganic sulfur exist as food additives in several forms, sulfur dioxide (E220), sulfites (E221-E227) and as a sulphated polysaccharide, carrageenan (E407). In Australia and Europe, food labelling requirements mandate only the labelling of added sulphites (in amounts > 10 mg/kg) in food product without specifying the amount used. Inorganic sulfur intake by foods and beverages has been showed to be six-fold higher in the western diet in comparison to a typical African rural diet[45,46]. However, food composition tables on sulfur-containing protein, inorganic sulfur and carrageenan are far from complete to adequately assess habitual intake of UC patients, to ensure successful design of the dietary therapy. More importantly, there are grounds that increasing a combination of fibre, rather than restricting total fibre, maybe an efficacious strategy for H_2S suppression whilst improving nutrient delivery to the colonic epithelium in UC. Resistant starch and fructo-oligosaccharides, whilst efficacious, are fermented in the proximal colon[10]. Hence, a strategy that will carry the fermentation of these fibres across the entire colon by combining with a minimally fermentable fibre is required.

Indeed, tolerability and the potential clinical effects of a dietary approach incorporating strategies discussed above have already been evaluated. In an open-label dietary advice study, Day *et al*[47] reported excellent tolerability of dietary strategy called the 4-strategies to a sulphide-reducing (4-SURE) diet by patients with mild to moderately active UC. This was despite the 38% increase in dietary fibre and the four-fold increase in resistant starch intake by these patients. Food-related quality of life also increased markedly. Whilst it was impractical to assess colonic H_2S in this study, markers of protein fermentation, namely faecal branched chain fatty acids were used as a surrogate. The significant reduction in faecal branched to short-chain fatty acid ratio following the 4-SURE study indicated that protein fermentation being the major pathway for luminal H_2S production was reduced. Whilst the study did not intend to primarily assess clinical end-points due to the uncontrolled study design, there were indicators for the diet to positively affect clinical outcomes and mucosal healing. Data supported by a significant reduction in faecal calprotectin. A second dietary approach, called the Ulcerative Colitis Exclusion Diet (UCED), also incorporated a similar exclusion of decreasing intake of total protein, sulphur-containing amino acids, food additives along with additional restrictions of animal and saturated fat, haeme, whilst increasing intake of tryptophan, pectin and resistant starch[48]. In a RCT comparing a combination of UCED with faecal transplant, faecal transplant or diet alone, Sarbagili Shabat *et al*[48] observed that clinical response and endoscopic remission were the greatest for the UCED diet. Furthermore, the promising outcomes of the UCED was supported by an earlier open-label study in paediatric patients with mild to moderate active UC on stable maintenance therapy, where the diet treatment showed that patients had a significant decrease in sulfur-containing amino acids consumption as well as a significant increase in total fiber consumption[49].

Whilst these proposed dietary approaches are not quite ready for clinical application until RCTs have been performed (currently underway) to replicate the promising findings, it does suggest that patients with active inflammation do tolerate a certain increase in high fibre foods and builds on the suggestion to minimise intake of processed foods. Moreover, the limitations of the available reported clinical trials targeting reduction of H_2S production as a treatment strategy for UC (Table 2) suggest the need for larger, high quality dietary studies incorporating gut microbiome composition and function assessment including changes in microbial H_2S metabolism.

BIOMARKERS FOR ASSESSING RESPONSE OF DIETARY THERAPY

It is key that a biomarker for assessing diet response is incorporated early on after dietary therapy is administered as a way of assessing whether the diet is achieving its intended mechanistic effect. An

Table 1 Content of sulfur, nitrate and nitrite in selected foods

Food category	Specific food	Sulfur amino acids (cysteine + methionine)[1], mg/100 g	Sulfates[2], mg/100 g/mL	Nitrate[3], mg/kg	Nitrite[3], mg/kg
High sulfur amino acids foods	Beef	239	-	-	-
	Chicken	291	-	-	-
	Turkey	269	-	-	-
	Tuna	268	-	-	-
	Prawns	189	-	-	-
	Eggs	162	-	-	-
	Cheese, hard	174	-	-	-
High sulfites foods	Dried apricots	-	300	-	-
	Dried apples	-	490	-	-
	Commercial bread	-	80-150	-	-
	Wine	-	38	-	-
High sulfates foods	Cabbage	-	84	-	-
	Broccoli	-	90	-	-
	Cauliflower	-	50	-	-
	Brussels sprouts	-	93	-	-
High nitrates foods	Lettuce	-	-	2351	-
	Celery	-	-	2110	-
	Spinach	-	-	1509	-
	Leek	-	-	841	-
High nitrites foods	Sausages, boiled	-	-	-	40
	Poultry meat	-	-	-	32
	Beef	-	-	-	59
	Bacon	-	-	-	86

[1]From reference Magee *et al*[46], 2004.
[2]From reference Florin *et al*[45], 1993.
[3]From reference Temme *et al*[44], 2011.

example of this is the reduction in breath hydrogen production after introduction of a diet low in fermentable carbohydrates as a biomarker of intervention success[50]. However, in the case of dietary approaches targeting colonic H_2S and NO, there are difficulties with accessing reliable measurement techniques for these volatile gases, particularly with *ex vivo* measurements often requiring freshly passed faecal samples[17,33], which introduces practical issues for trial patients. Currently, measurements for H_2S mainly involve faecal sulphide, urinary sulphate or breath H_2S[51]. Sensitivity of these measurements are impacted by its adsorption or susceptibility to oxidation, yielding low concentrations[51]. In contrast, the only reported assessment of luminal NO has been using direct sampling (*via* a tonometric balloon) and measurement *via* a rapid-response chemiluminescence technique[21]. While the method has good sensitivity, it is unknown whether this biomarker is directly responsive to alterations in dietary nitrate and nitrite intake. Finally, there is potential for direct intestinal gas sampling, such as the gas-sensing capsule[52], but these do not yet measure H_2S or NO. In the absence of reliable direct measurements, indirect assessments could target markers of protein fermentation for H_2S, quantification of sulphate- or sulphite-reducing bacteria which are dependent on availability of proteinaceous substrates for growth[53], and have capacities for denitrification and sulphate-reduction [54]. Hence, an effective biomarker for monitoring the success of sulphide- and NO-reducing dietary approaches remains elusive and is very much needed to support the development of the proposed dietary therapies. Therefore, as in most studies, assessment of dietary response is primarily assessed by different questionnaires such as dietary intake questionnaires, food-related quality of life or health-related quality of life questionnaire. Combined biomarker measurements with assessment by question-

Table 2 Summary of studies clinical outcomes by dietary interventions for ulcerative colitis as a possible strategy to modify hydrogen sulfide production

Ref.	Dietary intervention	Study design	Main outcomes	Limitations
Roediger[9], 1998	Low sulfur diet	Open-label, prospective pilot study. Patients were instructed to follow low sulfur diet + stable dose of salazopyrin for 12 mo (n = 4 adults)	All patients showed clinical and histological improvement and no relapse attacks were observed	Very small sample size
Bhattacharyya *et al*[43], 2017	No-carrageenan diet	Double-blind RCT: Carrageenan capsules versus placebo. Patients with remission were followed up until relapse or of 12 mo (n = 12 adults)	The carrageenan group demonstrated significant higher relapse rate and an increase in FC and IL-6 values from study onset	Small sample size in each group. The effects on the microbiome were not addressed and precise measurements of compliance with the diet were not performed
Chiba *et al*[58], 2019	Lacto-ovo-semivegetarian diet-PBD	Prospective single arm study. Patients were followed after induction therapy incorporating PBD (n = 92 children and adults)	The cumulative relapse rates at 1 and 5 yr were 14% and 27% respectively, which is indicated by the authors to be lower than those previously reported	Small sample size without control group. The mechanistic effect of the diet was not addressed
Sarbagili Shabat *et al*[48], 2022	UCED	Single-blind RCT in adults with active refractory UC: Group1: FT alone; group2: FT with UCED; group3: UCED alone. The primary endpoint was week 8 clinical remission (n = 51)	UCED alone demonstrated the greatest clinical and endoscopic remission rates compared to single donor FT with or without diet	Small sample size in each group. Eligibility criteria include patients with severe UC, of whom none obtain remission. The effects on the microbiome were not addressed
Sarbagili-Shabat *et al*[49], 2021	UCED	Open-label, prospective pilot study in children with active UC. The primary endpoint was week 6 clinical remission (n = 24)	UCED lead to 38% clinical remission and FC improvement	Small sample size without control group. The effects on the microbiome were not addressed
Day *et al*[47], 2022	4-SURE	Open-label, prospective pilot study in adults with active UC. The primary endpoint was week 8 tolerability (n = 28)	The 4-SURE diet was well tolerated and lead to 46% clinical response and 36% endoscopic improvement. Fecal excretion of SCFAs increased while BCFAs decreased	Changes in colonic H_2S not able to be measured. Lack of control and inadequate power for interpretation of secondary clinical end-points

RCT: Randomized controlled trial; FC: Fecal calprotectin; IL-6: Interleukin-6; PBD: Plant-based diet; UCED: Ulcerative colitis exclusion diet; FT: Faecal transplantation; UC: Ulcerative colitis; 4-SURE: 4 Strategies to SUlfide-Reduction; SCFA: Short chain fatty acid; BCFA: Branched chain fatty acid; H_2S: Hydrogen sulfide.

naires can be the ideal tool for estimating the effect of specific dietary exposure.

CONCLUSION

Microbial H_2S and NO metabolites have causative roles in the pathogenesis of UC *via* their damaging effects on the colonic epithelium. Modulation of their production within the colonic lumen in order to reduce colonic epithelial exposure to these luminal stressors presents an attractive therapeutic target that has yet to be adequately explored. The current evidence suggests that dietary manipulation is likely to be an effective strategy to modify colonic H_2S production whereas little is known regarding dietary modulation of NO. It is also clear that sulfur-containing amino acids are major substrates that promote H_2S production over inorganic sulphur but data has emerged suggesting that increasing fermentable fibre is highly efficacious in reducing H_2S production. These findings have been utilised to inform the design of multi-prong dietary approaches which have yielded promising therapeutic efficacy in mild to moderate active UC. However, key to advancing the success of this research is the urgent need for better technology to accurately assess luminal concentrations of these volatile gases. Finally, before implementation into dietary practice can be pursued, further investigations into their efficacy on altering disease activity using robust dietary trial designs (which are currently underway), expansion of food composition data and mechanisms of H_2S reduction are highly warranted.

FOOTNOTES

Author contributions: Yao CK and Sarbagili-Shabat C conducted the literature search; Yao CK devised headings for the article; and all authors drafted, wrote the article and approved of the final content.

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She also works in a department that financially benefits from the sales of a digital application and booklets on the low fermentable oligosaccharides, disaccharides, monosaccharides and polyols diet. Funds raised contribute to research of the Department of Gastroenterology and to the University. She does not receive personal remuneration. Sarbagili-Shabat C has no conflicts of interest to report.

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Country/Territory of origin: Australia

ORCID number: Chu K Yao 0000-0002-5301-2457.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Disease trends after *Helicobacter pylori* eradication based on Japanese nationwide claims and the health check-up database

Kazuhiro Mizukami, Kentaro Sugano, Tomomi Takeshima, Kazunari Murakami

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Kazuhiro Mizukami, Kazunari Murakami, Department of Gastroenterology, Oita University, Oita 8795593, Japan

Kentaro Sugano, Department of Medicine, Jichi Medical University, Tochigi 3290498, Japan

Tomomi Takeshima, Milliman Inc., Tokyo 1020083, Japan

Corresponding author: Kazuhiro Mizukami, MD, PhD, Associate Professor, Department of Gastroenterology, Oita University, 1-1 Idaigaoka, Hasama, Yufu, Oita 8795593, Japan. mizkaz0809@oita-u.ac.jp

Abstract

BACKGROUND

Helicobacter pylori (*H. pylori*) infection is a risk factor for many diseases, including peptic ulcer disease and gastric cancer. While *H. pylori* eradication therapy can prevent these diseases, potentially unfavorable effects of eradication therapy have also been reported in some diseases, such as gastroesophageal reflux disease (GERD), Barrett's esophagus (BE), inflammatory bowel disease (IBD), allergic diseases, and metabolic diseases. Consequently, both positive and negative impacts should be considered when assessing the effects of *H. pylori* eradication therapy.

AIM

To compare the incidence of these diseases before and after *H. pylori* eradication and to comprehensively assess its effects.

METHODS

This retrospective cohort study used a Japanese nationwide health claims database (April 2009-March 2020), developed by the Japanese Ministry of Health, Labour and Welfare. The database contained almost all health insurance claims data issued in Japan, and specific health check-up data for individuals who took the check-ups. Descriptive statistics were used for the analyses. Patients who received primary eradication therapy were defined as those prescribed medication for *H. pylori* eradication. New diagnoses, defined as incidence of upper gastrointestinal diseases and IBD, and prevalence of allergic diseases were compared before and after eradication. The incidence and prevalence of each disease were also compared between the 3-year period before eradication (from the 4th to the 2nd year prior to the year of eradication) and the 3-year period after

eradication (from the 1st to the 3rd year after the year of eradication) based on the age category and calendar year and month. Changes in body mass index and proportion of patients with metabolic syndrome (MS) were examined before and after eradication.

RESULTS

We identified 5219731 patients who received primary eradication therapy. The 65-69 years age group had the greatest number of patients in both sexes. There was no significant increase in the incidence of GERD after eradication when considering the effects of aging and reporting period. However, the incidence of BE was higher in the 3-year period after eradication than in the 3-year period before eradication for all age categories (0.02%-0.10% *vs* < 0.01%-0.05%). The incidence of IBD and prevalence of allergic disease were also higher after eradication. In contrast, the incidence of gastric and duodenal ulcers and gastritis was reduced after eradication. In patients with at least one entry of health check-up data (1701111 patients), the percentage of patients with MS showed a slight increase following eradication (11.0% in the year of eradication and 12.2% after 5 years).

CONCLUSION

The results suggest that *H. pylori* eradication therapy reduces peptic ulcers and gastritis; however, it is associated with increased incidence of several other chronic diseases.

Key Words: Administrative claims; Healthcare; Allergy; Eradication therapy; Gastroesophageal reflux disease; *Helicobacter pylori*; Inflammatory bowel disease

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Core Tip: While *Helicobacter pylori* (*H. pylori*) eradication can prevent certain diseases including peptic ulcer diseases and gastric cancer, unfavorable effects of eradication therapy have also been reported. We analyzed a Japanese nationwide health claims database containing almost all health insurance claims data to compare the incidence and prevalence of specific diseases before and after *H. pylori* eradication to comprehensively assess its effects. We identified 5219731 patients who received primary eradication therapy. *H. pylori* eradication drastically reduced peptic ulcers and gastritis but was associated with an increase in Barrett's esophagus, inflammatory bowel disease, allergic disease, and metabolic syndrome.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is a risk factor for many diseases, including peptic ulcers[1], gastric cancer[2], gastric mucosa-associated lymphoid tissue lymphoma[3], *H. pylori*-associated gastritis[4], idiopathic thrombocytopenic purpura[5], and iron deficiency anemia[6]. *H. pylori* eradication therapy can effectively prevent these diseases. However, unfavorable effects of eradication therapy have also been reported. For instance, the relationship between *H. pylori* infection and gastroesophageal reflux disease (GERD) is controversial. Some studies have suggested a lower incidence of GERD and its complications, such as Barrett's esophagus (BE), in patients with *H. pylori* infection[7-10]. A study reported that the prevalence of GERD increased in patients after successful *H. pylori* eradication and was comparable to that in patients without *H. pylori*[11]. However, some studies found no association between *H. pylori* eradication and GERD[12], while others reported that eradication is not associated with GERD development in dyspeptic patients but might pose a higher risk in patients with peptic ulcers[13]. An inverse association of *H. pylori* infection with some immune system diseases, including allergies[14-18] and inflammatory bowel disease (IBD)[18-22], has also been reported. Moreover, relationships between *H. pylori* infection and metabolic diseases and associated conditions such as obesity have also been described. *H. pylori* infection may increase the risk of dyslipidemia[23,24] and metabolic syndrome (MS)[25]. While favorable effects of *H. pylori* eradication on triglyceride and high-density lipoprotein cholesterol (HDL-C) levels[26] or dyslipidemia[27] have been reported, it has also been found that eradication therapy may increase the incidence of hyperlipidemia and obesity[28,29]. Therefore, both positive and negative impacts should be considered when assessing the effects of *H.*

pylori eradication therapy. A comprehensive evaluation of these effects in Japan, where large-scale eradication therapy was commenced earlier than it was in other countries due to its coverage by universal healthcare, might provide useful information for clinicians worldwide.

Herein, we performed a comprehensive analysis of the effects of *H. pylori* eradication therapy on various diseases. This study used a Japanese nationwide health claims database, the National Database of Health Insurance Claims and Specific Health Checkups of Japan (NDB), developed by the Japanese Ministry of Health, Labour and Welfare, containing almost all (≥ 95%) health insurance claims data issued in Japan[30]. In this database, health check-up data are also included for individuals who underwent specific health check-ups. We compared the development of diseases (defined as the first diagnosis) known to be related to *H. pylori* infection and eradication before and after eradication in patients who underwent primary *H. pylori* eradication therapy. We also assessed changes in body mass index (BMI) and MS development before and after eradication.

MATERIALS AND METHODS

Study design and data source

This claims-based study used NDB data[30] (April 2009-March 2020). The dataset contained medical claims data, diagnosis procedure combination (DPC) claims data (claims related to bundled payments during DPC hospitalization), and pharmacy claims data from patients who received primary *H. pylori* eradication therapy or those who had at least one diagnosis of GERD. Specific health check-up data available for patients who underwent check-ups included anthropometric data, laboratory values, and answers to questionnaires. The observation period was defined as the entire period of data availability for each patient.

Patients

Patients with a prescription for primary *H. pylori* eradication therapy covered by national health insurance were identified and designated in our study as those who received primary *H. pylori* eradication therapy. The three medications approved for this therapy are amoxicillin and clarithromycin with either a proton pump inhibitor (PPI) or potassium-competitive acid blocker (P-CAB); these can be prescribed as a combination of single drugs in the same month or as a combination pack (Supplementary Table 1).

Outcomes and analysis

We compared the incidences of GERD, BE, other upper gastrointestinal diseases [including some diseases of the digestive system other than those categorized as International Statistical Classification of Diseases and Related Health Problems, 10th revision (ICD-10) code K92], and IBD, and the prevalence of allergic diseases before and after primary *H. pylori* eradication therapy (see Supplementary Table 2 for definition of the diseases). Furthermore, IBD was classified into Crohn's disease (CD) and ulcerative colitis (UC) according to the ICD-10 classification. Other upper gastrointestinal diseases were classified into 10 types [esophagitis, other diseases of the esophagus, gastric ulcer (GU), duodenal ulcer (DU), peptic ulcer, site unspecified, anastomotic ulcer, gastritis and duodenitis, functional dyspepsia (FD), other diseases of the stomach and duodenum, and other diseases of the digestive system] according to the ICD-10 classification, and allergic diseases were classified into five types (allergy, pollinosis, asthma, hypersensitivity, and atopy) by disease name (Supplementary Table 2). It should be noted that since allergic diseases were initially defined according to the ICD-10 classification and atopic asthma was classified into the asthma group, the atopy group included only atopic cough. The month of primary eradication therapy was defined as the earliest month in which primary eradication medications were prescribed for each patient. The term 'year of eradication' refers to the year corresponding to this month. The incidence of a disease was calculated on the basis of the earliest first diagnosis of each disease, which was identified using the record of the first diagnosis date by the medical institution on medical claims or DPC claims in the database.

The incidence and prevalence of each disease was calculated for each elapsed year from the eradication, which was calculated as the integer part of the difference of the time and the time of the eradication. If the difference was negative, the elapsed year was the integer part minus one, as in SAS (version 9.4, SAS Institute, Cary, NC), the integers of the negative numbers were rounded up.

We also compared the incidence and prevalence of each disease between the 3-year period before eradication (from the 4th to 2nd year prior to the year of eradication) and the 3-year period after eradication (from the 1st to 3rd year after the year of eradication) based on the age category and calendar year and month, represented by calendar month and year from July 2014 to June 2018.

To assess GERD status in patients with and without *H. pylori* eradication, we compared treatments in patients who received eradication therapy before GERD development with those who did not undergo eradication therapy during the observation period. Patients with available health check-up data for the year of the first GERD diagnosis were included in this analysis (eradication group), and the data were used to develop a propensity score (PS). Patients in the non-eradication group were identified from the

patients with GERD, those who did not undergo eradication therapy, and those who had their health check-up data. The patients identified in the non-eradication group were those whose sex, age at the first GERD diagnosis, and the month of the first GERD diagnosis matched with those of anyone in the eradication group. After adjusting for confounding factors using the PS, the types of drugs prescribed for GERD were compared by year from first GERD diagnosis and by calendar year between the eradication and non-eradication groups. The PS was estimated using a logistic regression model with eradication (dummy variable) as the explained variable, and age, sex, and specific health check-up data (BMI, abdominal circumference, systolic blood pressure, diastolic blood pressure, triglyceride, HDL-C, low-density lipoprotein cholesterol, aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transpeptidase, fasting blood glucose, and hemoglobin A1c) as explanatory variables. The PS was divided into four classes, and patients within each group were assigned the same weight to make the total weight comparable between groups in each PS class. The following drug classes for GERD treatment were analyzed: Histamine H_2 receptor antagonist, P-CAB, PPI, and others (Supplementary Table 3). In patients who received GERD treatment in the month of first diagnosis, the period of continuous GERD treatment was compared between patient groups after adjustment using the Kaplan-Meier method with the log-rank test (in this test, $P < 0.05$ was considered statistically significant). Treatment was defined as any treatment with no gap exceeding 3 mo.

Changes in BMI and proportion of patients with MS were examined before and after eradication in patients for whom these data of specific health check-up were available at least once or yearly for the 3 years before and after the year of eradication. If multiple values were recorded for a patient in the same year, the average value was used. Patients with MS were defined as those with an abdominal circumference of ≥ 85 cm in men or ≥ 90 cm in women, and who met at least two of the following criteria: (1) Triglyceride ≥ 150 mg/dL or HDL-C < 40 mg/dL; (2) Systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg; and (3) Fasting plasma glucose ≥ 110 mg/dL[31]. We used the statistical software packages SAS for the analyses. In the analyses using the specific health check-up data, missing values were imputed based on all other factors using the MI statement in PROC MI of the SAS software.

Ethical approval

This study was approved by the Ethics Committee of Oita University, Faculty of Medicine (No. 1692). All procedures followed were in accordance with the World Medical Association's Declaration of Helsinki (1964, and its later amendments). Informed consent was not obtained because this study used anonymized claims data.

RESULTS

Overview of disease developments

The dataset included 5219731 patients who received primary *H. pylori* eradication therapy. Maximum number of patients were in the 65-69 years age bracket at the time of eradication. There were more men aged < 65 years, but more women aged ≥ 65 years (Figure 1).

The incidences of upper gastrointestinal diseases and IBD were highest in the year of eradication and the preceding year, respectively (Table 1). After eradication, the incidences of GERD and IBD decreased yearly, reaching similar levels to those before eradication. The incidences of other upper gastrointestinal diseases markedly decreased after eradication. In contrast, the incidence of BE increased after eradication. The prevalence of allergic diseases increased in the years before eradication and continued to increase after eradication; the prevalence at 5 years after eradication was almost double the prevalence approximately 10 years before eradication (Table 1).

GERD

The incidence of GERD in the 3-year period before eradication was similar to or slightly higher than that in the period after eradication for all age groups, and it tended to increase with age regardless of eradication status (Figure 2A). When data were analyzed according to the calendar year and month, the incidence was higher after eradication, although this difference disappeared in later years (Figure 2B).

When comparing GERD treatment status between the eradication group (76367 patients) and non-eradication group (1008539 patients), there was no remarkable difference in the trend of GERD medication by year from first GERD diagnosis or calendar year, although the percentage of patients receiving each type of GERD drug was slightly higher in the eradication group (Supplementary Figure 1). In patients who received GERD medication in the month of the first GERD diagnosis (72170 and 920794 patients in the eradication and non-eradication groups, respectively), the continuous treatment period was significantly longer in the eradication group ($P < 0.01$) (Supplementary Figure 2).

BE

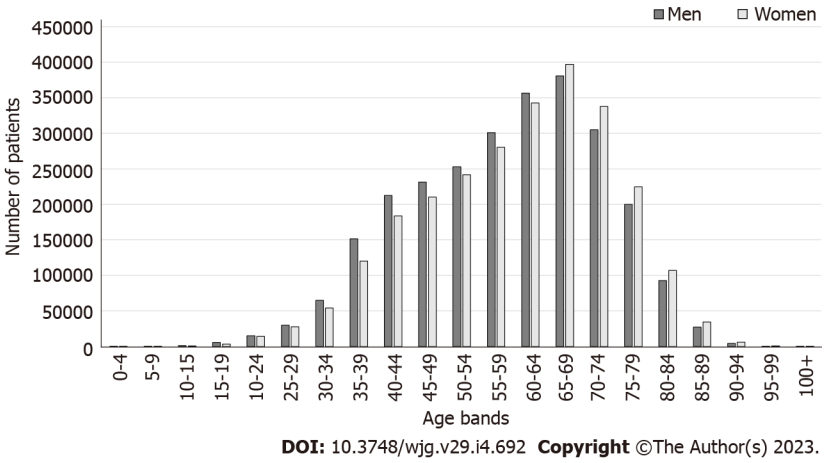
The incidence of BE was higher in the 3-year period after eradication than in the period before eradication for all age categories (Figure 3A), and throughout the observation period (Figure 3B). The

Table 1 Incidence or prevalence of each disease by year from primary *Helicobacter pylori* eradication therapy

Year	Denominator (patient-year)	Average age	Upper gastrointestinal diseases			IBD	Allergy
			GERD	Barrett's esophagus	Other upper gastrointestinal diseases		
-10	1146497	48.9	2.31% (2.28%, 2.33%)	0.004% (0.002%, 0.005%)	11.45% (11.39%, 11.51%)	0.029% (0.026%, 0.032%)	17.5% (17.4%, 17.5%)
-9	1943505	50.1	2.55% (2.52%, 2.57%)	0.006% (0.005%, 0.008%)	11.10% (11.05%, 11.14%)	0.028% (0.025%, 0.030%)	20.0% (19.9%, 20.0%)
-8	2761765	51.3	2.75% (2.73%, 2.77%)	0.007% (0.006%, 0.008%)	10.57% (10.54%, 10.61%)	0.026% (0.024%, 0.028%)	21.3% (21.2%, 21.3%)
-7	3364755	52.5	3.02% (3.00%, 3.04%)	0.010% (0.009%, 0.011%)	9.89% (9.86%, 9.92%)	0.027% (0.025%, 0.029%)	23.1% (23.1%, 23.2%)
-6	4001760	53.7	3.18% (3.16%, 3.19%)	0.011% (0.010%, 0.012%)	9.27% (9.24%, 9.30%)	0.027% (0.025%, 0.028%)	24.4% (24.3%, 24.4%)
-5	4520587	54.9	3.38% (3.37%, 3.40%)	0.015% (0.014%, 0.016%)	8.67% (8.64%, 8.70%)	0.028% (0.026%, 0.029%)	25.7% (25.7%, 25.7%)
-4	4746859	55.9	3.48% (3.47%, 3.50%)	0.018% (0.017%, 0.019%)	7.54% (7.51%, 7.56%)	0.027% (0.025%, 0.028%)	27.4% (27.3%, 27.4%)
-3	4880888	56.9	3.58% (3.56%, 3.59%)	0.023% (0.022%, 0.024%)	6.50% (6.48%, 6.53%)	0.027% (0.026%, 0.028%)	28.8% (28.7%, 28.8%)
-2	5025747	57.9	3.80% (3.78%, 3.81%)	0.030% (0.028%, 0.031%)	6.04% (6.02%, 6.06%)	0.029% (0.027%, 0.030%)	30.3% (30.3%, 30.4%)
-1	4732998	58.8	11.01% (10.99%, 11.04%)	0.164% (0.161%, 0.168%)	19.31% (19.27%, 19.35%)	0.046% (0.044%, 0.048%)	33.7% (30.3%, 30.4%)
0	4892585	59.8	11.33% (11.30%, 11.36%)	0.223% (0.219%, 0.227%)	16.48% (16.45%, 16.51%)	0.053% (0.051%, 0.055%)	34.7% (34.7%, 34.8%)
1	4137930	61.0	3.84% (3.82%, 3.86%)	0.083% (0.080%, 0.086%)	0.15% (0.15%, 0.16%)	0.034% (0.032%, 0.036%)	35.0% (34.9%, 35.0%)
2	3344893	62.2	3.30% (3.28%, 3.32%)	0.080% (0.077%, 0.083%)	0.12% (0.12%, 0.13%)	0.030% (0.028%, 0.032%)	35.7% (35.7%, 35.8%)
3	2520763	63.4	2.98% (2.96%, 3.00%)	0.076% (0.072%, 0.079%)	0.10% (0.09%, 0.10%)	0.030% (0.028%, 0.032%)	36.7% (36.7%, 36.8%)
4	1900509	64.5	2.76% (2.74%, 2.78%)	0.076% (0.072%, 0.080%)	0.09% (0.08%, 0.09%)	0.028% (0.026%, 0.031%)	36.5% (36.4%, 36.6%)
5	1275881	65.4	2.51% (2.49%, 2.54%)	0.073% (0.069%, 0.078%)	0.07% (0.07%, 0.07%)	0.028% (0.025%, 0.031%)	38.6% (38.5%, 38.7%)

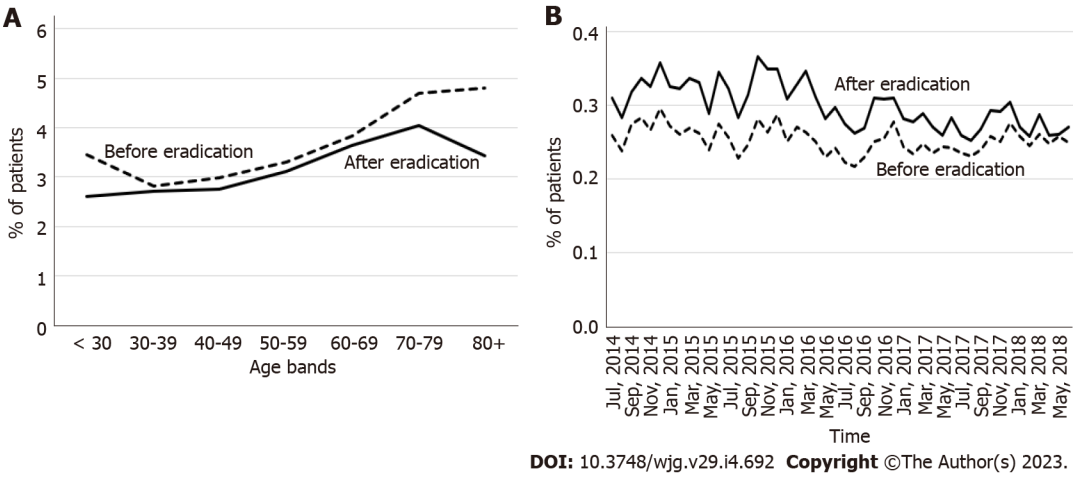
The prevalence of allergic diseases and incidence of the other diseases are shown with 95% confidence intervals (lower limit, upper limit). Year 0 is the year of primary eradication, and negative values represent years before primary eradication. The elapsed year from the eradication was calculated as the integer part of the difference in the year and the time of the eradication. If the difference was negative, the elapsed year was the integer part minus one, as in SAS, the integers of the negative numbers were rounded up. GERD: Gastroesophageal reflux disease; IBD: Inflammatory bowel disease.

incidence of BE increased with age regardless of *H. pylori* eradication status in patients < 70 years of age and decreased thereafter beyond the age of 70 years (Figure 3A).



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Figure 1 Number of patients who received primary *Helicobacter pylori* eradication therapy, by sex and age group at the time of eradication.



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Figure 2 Incidence of gastroesophageal reflux disease in the 3-year period before the year of eradication and after the year of eradication. A: By age categories; B: By calendar year and month.

Other upper gastrointestinal diseases

The incidence of other upper gastrointestinal diseases was lower in the 3-year period after eradication than in the period before eradication for all age categories (Supplementary Figure 3A), and throughout the study period (Supplementary Figure 3B).

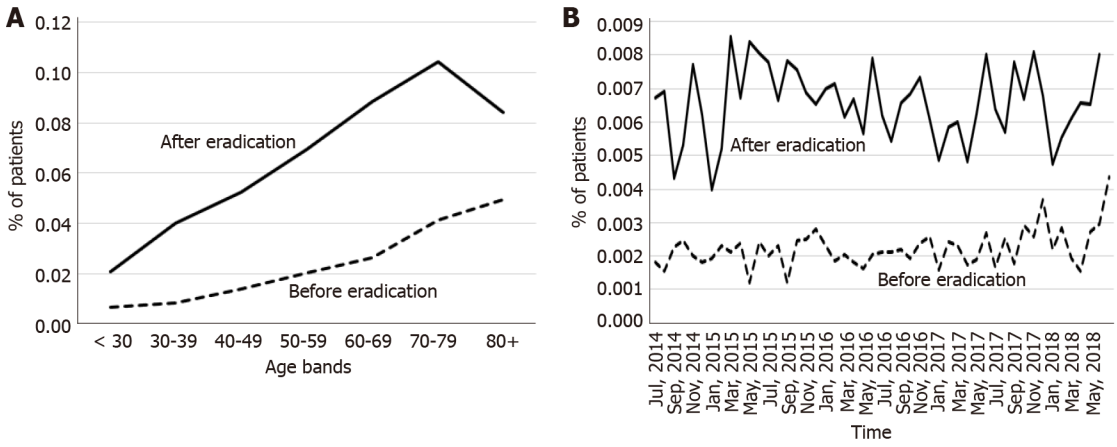
Gastritis and duodenitis showed the highest incidences before eradication, and their incidences decreased after eradication (Supplementary Figure 4). The incidences of GU and DU were also higher before eradication than after eradication (Supplementary Figure 4). The incidences of other diseases, including FD, esophagitis, and other diseases of the esophagus, increased with time and were higher after eradication than before (Supplementary Figure 4). Of note, the overall incidence of these diseases was lower than that of the other upper gastrointestinal diseases.

IBD

The incidence of CD was higher in the 3-year period after eradication than in the period before eradication for younger age categories. This difference decreased with increasing age, particularly for patients aged ≥ 60 years (Figure 4A). The incidence of UC was higher in the 3-year period after eradication than in the period before eradication for patients aged ≥ 30 years (Figure 4B). For both diseases, the incidence tended to decrease with increasing age up to 50-59 years; the incidence of CD was very low for this and older age groups (Figures 4A and B).

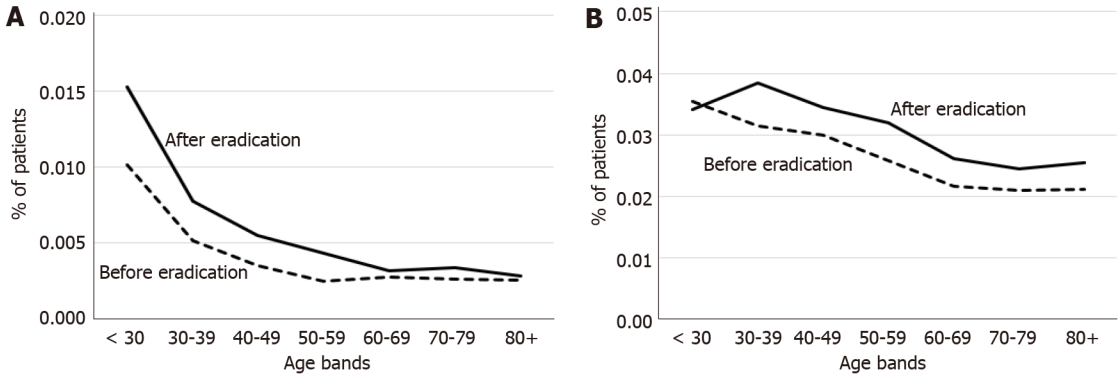
Allergic diseases

The prevalence of allergic diseases was higher in the 3-year period after eradication than in the period before eradication for all age categories (Figure 5A), and throughout the entire study period (Figure 5B).



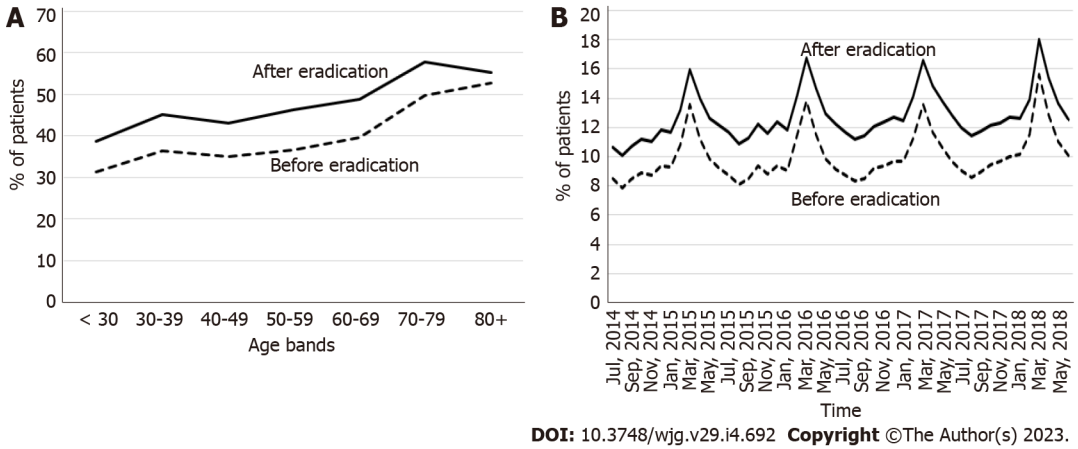
DOI: 10.3748/wjg.v29.i4.692 **Copyright** ©The Author(s) 2023.

Figure 3 Incidence of Barrett's esophagus in the 3-year period before the year of eradication and after the year of eradication. A: By age category; B: By calendar year and month.



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Figure 4 Incidence of inflammatory bowel disease in the 3-year period before the year of eradication and after the year of eradication by age category. A: Crohn's disease; B: Ulcerative colitis.



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Figure 5 Prevalence of allergic diseases in the 3-year period before the year of eradication and after the year of eradication. A: By age category; B: By calendar year and month.

The prevalence of allergic diseases tended to be higher in older patients (Figure 5A); moreover, periodic seasonal changes were observed, with a higher incidence in early spring (Figure 5B), both before and after eradication. The prevalence of all types of allergies increased after eradication (Supplementary Figure 5).

Changes in BMI and MS

At least one set of data regarding BMI was available for 1701111 patients during the observation period, and 100954 patients had BMI data available for every year of the 3 years before and after the year of eradication. Among patients with at least one data point, the average BMI slightly decreased toward the year of eradication, following which it slightly increased (Figure 6A). This increase was even more remarkable among those with complete BMI data in the 3 years before and after eradication.

In all patients with health check-up data available at least once (1701111 patients), the percentage of patients with MS slightly increased following eradication (Figure 6B). An even larger increase after eradication was observed in patients with all data from 3 years before and after eradication.

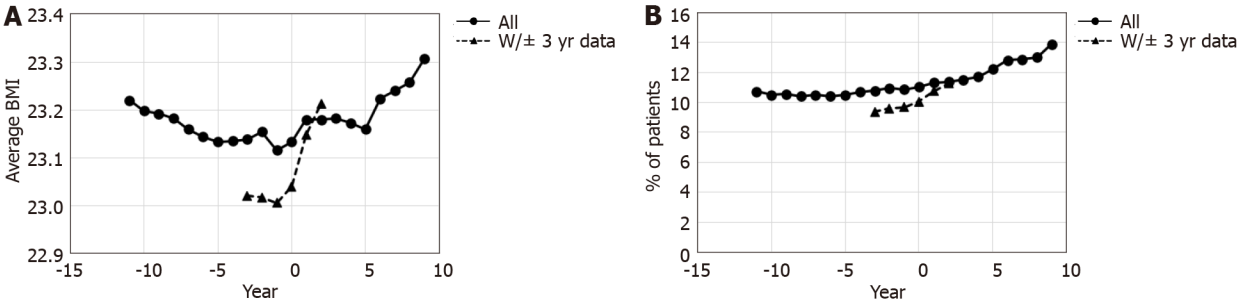
DISCUSSION

This study comprehensively analyzed the effects of *H. pylori* eradication, including both favorable and unfavorable aspects, in Japan. The database used in this study is a nationwide claims database comprising data from almost the entire Japanese population, including more than 5 million people who received primary *H. pylori* eradication therapy. While favorable effects on GU, DU, and gastritis were confirmed, the treatment could result in the development of several concerning diseases. In particular, the possibility of an increase in allergic diseases is a new finding that has not been reported in the past.

When the effect of aging and reporting period was considered, there was no significant increase in the incidence of GERD after eradication. Previous studies reported conflicting results regarding the relationship between *H. pylori* infection and GERD development. Among Japanese *H. pylori*-positive patients, the odds ratio (OR) for GERD development is reported to be 0.35-0.50[10,32,33]. The lower rates of GERD development in patients with *H. pylori* is reportedly due to the suppression of gastric acid production caused by *H. pylori* infection. However, a systematic review based on randomized controlled trials (RCTs) of patients who underwent *H. pylori* eradication indicated no significant increase in GERD occurrence at 6, 12, and 24 mo following eradication[12]. No significant effects of *H. pylori* eradication on GERD development were suggested by another systematic review analyzing RCTs[34]. In addition, another RCT showed that *H. pylori* eradication did not affect the recurrence rate of GERD after eradication[35]. In contrast, an increased risk following eradication therapy was suggested by a meta-analysis of cohort studies and RCTs[36]. These contradictory results might be due to differences in baseline diseases and study design. In fact, another systematic review suggested that eradication is not associated with GERD development in dyspeptic patients, whereas an association in patients with peptic ulcers is suggested by results of cohort studies but not of RCTs[13]. Collectively, the findings of previous reports together with our results suggest the lack of an association between *H. pylori* eradication and GERD development. Moreover, the type of GERD medication did not differ between patients with and without eradication. This suggests that a change in GERD treatment including a switch to more powerful P-CAB or an increase in dosage of PPI is not required following eradication.

After eradication, a higher incidence of BE was observed in all age groups and over time. A previous case-control study reported a lower risk of BE in patients with *H. pylori* infection, with an overall OR of 0.55 [95% confidence interval (CI): 0.35-0.84] after adjusting for age and white race, as well as an OR of 0.28 (0.15-0.50) in those with corpus atrophy or antisecretory drug use[37]. The diagnostic criteria of BE is different in Japan and Western countries, so direct comparisons are difficult[38]. However, a possible explanation for the higher incidence of BE may have to do with the palisade vessel at the distal esophagus, a landmark of the gastroesophageal junction used in Japan, being more clearly identifiable by endoscopy after eradication, as *H. pylori* may colonize the esophageal mucosa, causing inflammation [39,40]. After eradication of *H. pylori*, small areas of columnar metaplasia may become visible due to resolution of mucosal inflammation. Utilization of advanced image-enhanced endoscopy, such as linked color imaging may also contribute to a higher diagnostic performance for BE[41]. Notably, BE lesions in Japanese patients were very short (< 1 cm) in most cases, and patients with ≥ 3-cm lesions were rare (approximately ≤ 1%)[42]. A previous Japanese case report described a case of esophageal adenocarcinoma after *H. pylori* eradication[43]. However, a Swedish cohort study indicated no evidence of an increased risk of BE or esophageal adenocarcinoma after *H. pylori* eradication[44]. Further research, including an evaluation of the characteristics of BE and its association with cancer, is warranted to evaluate the true effects of eradication on BE in Japanese patients.

Both age-dependent and monthly analyses suggest lower incidences of other upper gastrointestinal diseases following eradication therapy. Among these diseases, GU, DU, and gastritis and duodenitis (analyzed together) were the most common before eradication. The effect of *H. pylori* eradication on the risk of GU, DU, and gastritis has been previously established[1,4]; therefore, the reduced incidence of these diseases after eradication was not surprising. Further, we found an increase in FD, as recorded in the claims data, after eradication (Supplementary Figure 3), contrary to previous meta-analysis on RCTs reporting improvement or no change after eradication[45-47]. This discrepancy may be explained by our insurance claim system. For patients with dyspepsia symptoms and *H. pylori* infection, their diagnosis would be *H. pylori*-positive gastritis, which allows them to receive eradication therapy under insurance coverage. However, if they still had dyspeptic symptoms after successful eradication, their diagnosis



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Figure 6 Change in the average body mass index and percentage of patient with metabolic syndrome before and after the year of eradication. A: Average body mass index; B: Percentage of patients with metabolic syndrome. Year 0 is the year of primary eradication, and negative values represent years before primary eradication. BMI: Body mass index.

would be converted to FD, thereby increasing the number of patients with this label.

A protective effect of *H. pylori* infection against IBD has been suggested by an observational cohort study[48] and meta-analyses[19-21]. This relationship is explained by the effects of *H. pylori* infection on the immune system[18]. *H. pylori* infection increases gastric mucosal expression of a regulatory T cell marker, forkhead box P3, which decreases inflammatory Th1/Th17 responses and leads to suppression of inflammatory diseases including IBD[18,21]. Recently, metagenomic studies demonstrated an uneven recovery of the human gut microbiome after treatment with antibiotics[49,50], and the use of antibiotics was associated with an increased risk of developing both new-onset CD and UC. This risk was highest in the 1st year after antibiotic intake[51]. However, a higher incidence after eradication was also observed in all ages when comparing the 3-year period before and after eradication, and the incidence of CD and UC was higher in younger patients. In previous reports, eradication was not associated with an increased risk of IBD[52], or numerically, but not significantly increased[53]. Meanwhile, a significant increase in the risk of autoimmune diseases including IBD after eradication has also been reported[54]. A case-control study using Swedish national registry data showed that a history of antibiotic use is associated with IBD development, and that dispensation of multiple antibiotics increases the risk[55]. Our results are therefore consistent with the Swedish national registry data and should be investigated in other populations.

An inverse correlation of *H. pylori* infection with allergy has been reported in Japan[17] and other countries, and several mechanisms have been proposed[14-16]. *H. pylori* neutrophil-activating protein (HP-NAP), a virulence factor of *H. pylori*, reportedly stimulates neutrophils, increasing their production of oxygen radicals and adhesion to endothelial cells[56]. HP-NAP was shown to inhibit eosinophil infiltration and serum immunoglobulin E production in the lung, resulting in inhibition of interleukin (IL)-4 and IL-5 production in a mouse model[57]. These immunological changes in response to *H. pylori* eradication may be associated with the development of allergic diseases, as supported by our own findings. While the estimated number needed to treat for 3 years before and after eradication was calculated to be 58.8 for GU, 714.3 for DU, and 19.2 for gastritis and duodenitis, the estimated number needed to harm (NNH) for this period was found to be 12.3 for allergic diseases (unpublished data); this highlights the necessity of monitoring the effects of eradication therapy on allergy. Nevertheless, the effects of eradication therapy on chronic urticaria remain controversial; eradication therapy is recommended for those with chronic urticaria in Japanese guidelines[58]. Further large-scale prospective studies are warranted to understand the association between eradication and allergic diseases.

There are many reports on the relationship between *H. pylori* eradication and BMI and MS (Supplementary Table 4). Previous reports indicate that eradication generally increases BMI. An RCT conducted in South West England including *H. pylori*-infected patients with an average BMI of approximately 27 kg/m^2 reported an increase in BMI after eradication[59]. The increase in BMI after eradication is reportedly caused by the improvement of dyspepsia symptoms[59] and effects on the hormonal systems *via* ghrelin and leptin, which regulate food intake and appetite[24,59].

On the other hand, controversy remains about MS. Previous reports, including a Japanese study before 2010, have reported that dyslipidemia is exacerbated by eradication. However, recent reports have suggested that the factors that make up MS, especially dyslipidemia and diabetes, tend to improve after eradication (Supplementary Table 4). Recent Korean studies examining patients with an average BMI of approximately 24 kg/m^2 suggested an association between *H. pylori* infection and the risk of dyslipidemia[24,27], as well as a decrease in this risk after successful eradication[27]. The difference between the previous reports and our results may be due to the difference in the background of the population and the length of the observation period. Dyslipidemia is greatly affected not only by *H. pylori* status but also by age, sex, socioeconomic status, BMI, smoking status, diet, alcohol consumption, and exercise. Recently, changes in the gut microbiota after eradication and its effects on metabolism

have been discussed[26,60], and further elucidation of this pathophysiology is awaited.

Limits of the study

To our knowledge, this is the first study to investigate the effects of *H. pylori* eradication on a comprehensive disease spectrum in one of the largest target populations with a long observation period. However, this study has several limitations. First, we defined patients who received *H. pylori* eradication therapy based on prescription data. As such, there are no data about actual drug intake and the results of eradication success. However, we can assume that eradication rate is approximately 80% with PPI-based triple therapy, and 90% with the use of P-CAB-based triple therapy[61]. For those who could not achieve eradication, nearly 100% success can be achieved with a secondary regimen with metronidazole. Second, disease incidences were based on records of the diagnoses; thus, it was not possible to confirm the true onset of the development of the disease. In addition, some patients may have been diagnosed at the time of the medical visit or examination for eradication treatment, as suggested by the highest incidence of gastrointestinal diseases around the year of eradication. Consequently, the incidence might be overestimated around the year of eradication or later. Furthermore, it is possible that some diagnoses recorded in the claims based on drug prescriptions or diagnostic tests could be tentative or even false, although regular audit of the claims is done. In order to reduce such short-term influences, we also analyzed changes of disease incidence in a longer time-span. Third, although the NDB database is comprehensive, we only included a limited subset of patients when assessing the GERD status in patients with and without eradication, (only patients having health check-up data in the year of first GERD diagnosis were included) or when evaluating its effects on BMI and MS (only patients having health check-up data within 3 years before and after eradication were included). This affects the generalizability of these analyses. Finally, these findings may not be applicable to other countries, particularly those with different dietary habits and physical constitutions.

CONCLUSION

Our data obtained from a nationwide Japanese claims and health check-up database suggest an increased development of BE, IBD, allergic diseases, BMI, and MS, but not of GERD after *H. pylori* eradication. Although we observed a drastic decrease in the incidence of GU, DU, and gastritis, a large increase in allergic diseases may cancel out these beneficial effects, considering NNH. We believe that a comprehensive long-term assessment of the treatment effects, considering both favorable and unfavorable outcomes, is necessary for evaluating the true value of *H. pylori* eradication.

ARTICLE HIGHLIGHTS

Research background

Helicobacter pylori (*H. pylori*) eradication therapy can prevent some diseases, including peptic ulcer disease and gastric cancer. However, potentially unfavorable effects of eradication therapy have also been reported for some diseases, such as gastroesophageal reflux disease (GERD), Barrett's esophagus (BE), inflammatory bowel disease (IBD), allergic diseases, and metabolic diseases. Consequently, both positive and negative impacts should be considered when assessing the effects of *H. pylori* eradication therapy.

Research motivation

This study compared the incidence of some diseases before and after *H. pylori* eradication therapy in order to assess the positive as well as negative effects of the therapy. The comprehensive evaluation of these effects in Japan, where large-scale eradication therapy was commenced earlier than it was in other countries because of coverage by universal healthcare, might provide useful information for clinicians worldwide.

Research objectives

The objective of this study was to compare the incidence of some diseases, which appear to be associated with *H. pylori* eradication therapy, before and after the eradication in order to obtain a comprehensive overview of the treatment effects.

Research methods

This study used a Japanese nationwide health claims database, the National Database of Health Insurance Claims and Specific Health Checkups of Japan (NDB; April 2009-March 2020), developed by the Japanese Ministry of Health, Labour and Welfare. The database contains almost all (≥ 95%) health insurance claims data issued in Japan as well as health check-up data for individuals who underwent

specific health check-ups. Patients with a prescription for primary *H. pylori* eradication therapy covered by national health insurance were analyzed as those who received primary *H. pylori* eradication therapy. The incidences of GERD, BE, other upper gastrointestinal diseases, and IBD; the prevalence of allergic diseases and metabolic syndrome (MS); and changes in body mass index (BMI) were examined before and after primary *H. pylori* eradication therapy.

Research results

In total, 5219731 patients who received primary eradication therapy were identified in the database. There was no significant increase in the incidence of GERD after eradication when considering the effects of aging and the reporting period. The incidence of BE was higher in the 3-year period after eradication than in the period before eradication for all age categories. The incidence of IBD and prevalence of allergic disease were also higher after eradication. In contrast, the incidences of gastric and DUs and gastritis were decreased after eradication. Among patients with at least one entry of health check-up data (1701111 patients), the percentage of patients with MS showed a slight increase following eradication (11.0% in the year of eradication and 12.2% after 5 years). Because this study only used information recorded in the claims database, the disease incidences were based on records of the diagnoses, and it was not possible to confirm the true onset of the development of the disease. The accuracy of the records of diagnoses also affected the results of this study.

Research conclusions

To our knowledge, this is the first study to examine the effects of *H. pylori* eradication therapy using a large-scale database in Japan. The results suggest that there is an increase in BMI and the development of BE, IBD, allergic diseases, and MS, but not in the development of GERD, after *H. pylori* eradication therapy. Although the treatment can drastically decrease the incidences of gastric and duodenal ulcers and gastritis, a considerable increase in allergic diseases may cancel out these beneficial effects.

Research perspectives

A comprehensive, long-term assessment of the treatment effects, with consideration of both favorable and unfavorable effects, is necessary for evaluating the true value of *H. pylori* eradication therapy.

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FOOTNOTES

Author contributions: Sugano K contributed to the conception of the study; Sugano K, Takeshima T, and Murakami K contributed to the design of the study; Mizukami K, Sugano K, Takeshima T, and Murakami K contributed to the interpretation of data; Mizukami K and Takeshima T wrote the article; Sugano K and Murakami K critically reviewed the manuscript.

Institutional review board statement: The study was reviewed and approved for publication by the Ethics Committee of Oita University, Faculty of Medicine (No. 1692).

Informed consent statement: Informed consent was not obtained because this study used anonymized claims data.

Conflict-of-interest statement: Sugano K received lecture fees from Takeda Pharma. Inc and he is an advisor of Phathom Co. Takeshima T is an employee of Milliman Inc., which provides consulting service for biomedical companies. Mizukami K and Murakami K declare that they have no conflicts of interest.

Data sharing statement: Data will not be made available to other researchers because access to the raw data is strictly limited to authorized researchers, and the raw data and interim analysis data must be deleted after the authorized research period. Analytic methods and analysis results approved for publication by the Ministry of Health, Labour and Welfare in Japan will be made available upon reasonable request.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

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Country/Territory of origin: Japan

ORCID number: Kazuhiro Mizukami 0000-0002-3148-8884; Kentaro Sugano 0000-0002-8578-2974; Tomomi Takeshima 0000-0002-0572-5145; Kazunari Murakami 0000-0003-2668-5039.

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ORIGINAL ARTICLE

Retrospective Study

Diagnostic and economic value of carcinoembryonic antigen, carbohydrate antigen 19-9, and carbohydrate antigen 72-4 in gastrointestinal cancers

Hai-Ning Liu, Can Yao, Xiao-Fan Wang, Ning-Ping Zhang, Yan-Jie Chen, Dong Pan, Guo-Ping Zhao, Xi-Zhong Shen, Hao Wu, Tao-Tao Liu

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Hai-Ning Liu, Xiao-Fan Wang, Ning-Ping Zhang, Yan-Jie Chen, Xi-Zhong Shen, Hao Wu, Tao-Tao Liu, Department of Gastroenterology and Hepatology, Zhongshan Hospital, Fudan University, Shanghai 200032, China

Can Yao, Department of Gastroenterology and Hepatology, Minhang District Central Hospital, Fudan University, Shanghai 201199, China

Dong Pan, Department of Internet Technology Center, Zhongshan Hospital, Fudan University, Shanghai 200032, China

Guo-Ping Zhao, Chinese National Human Genome Center at Shanghai, Zhujiang Hospital, Central Lab, Institute of Plant Physiology and Ecology, Shanghai Institute for Biological Sciences, Shanghai 200032, China

Corresponding author: Tao-Tao Liu, MD, Doctor, Department of Gastroenterology and Hepatology, Zhongshan Hospital, Fudan University, No. 180 Fenglin Road, Shanghai 200032, China. liu.taotao@zs-hospital.sh.cn

Abstract

BACKGROUND

The diagnostic and economic value of carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9) and CA72-4 for gastrointestinal malignant tumors lacked evaluation in a larger scale.

AIM

To reassess the diagnostic and economic value of the three tumor biomarkers.

METHODS

A retrospective analysis of all 32857 subjects who underwent CEA, CA19-9, CA72-4, gastroscopy and colonoscopy from October 2006 to May 2018 was conducted. Then, we assessed the discrimination and clinical usefulness. Total cost, cost per capita and cost-effectiveness ratios were used to evaluate the economic value of two schemes (gastrointestinal endoscopy for all people without blood tests *vs* both gastroscopy and colonoscopy when blood tests were positive).

RESULTS

The analysis of 32857 subjects showed that CEA was a qualified biomarker for colorectal cancer (CRC), while the diagnostic efficiencies of CA72-4 were catastrophic for all gastrointestinal cancers (GICs). Regarding early diagnosis, only CEA could be used for early CRC. The combination of biomarkers didn't greatly increase the area under the curve. The economic indicators of CEA were superior to those of CA19-9, CA72-4 and any combination. At the threshold of 1.8 μg/L to 10.4 μg/L, all four indicators of CEA were lower than those in the scheme that conducted gastrointestinal endoscopy only. Subgroup analysis implied that the health checkup of CEA for people above 65 years old was economically valuable.

CONCLUSION

CEA had qualified diagnostic value for CRC and superior economic value for GICs, especially for elderly health checkup subjects. CA72-4 was not suitable as a diagnostic biomarker.

Key Words: Diagnostic test; Economic analysis; Cost-effectiveness analysis; Decision curve analysis

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Core Tip: This is a retrospective study to reassess the diagnostic and economic value of traditional tumor biomarkers carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9) and CA72-4 for gastrointestinal malignant tumors in a large sample with novel indicators. Instead of increasing the diagnostic value, CA72-4 should be removed from the list of the health checkup items to avoid the waste of social medical resources for CEA were superior to those of CA19-9, CA72-4 or any other combinations in which it could be applied for early colorectal cancer and a health checkup of CEA for people above 65 years old was economically valuable.

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INTRODUCTION

Blood carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) are widely used as classic diagnostic markers for malignant tumors, and they are recommended by several clinical guidelines for gastrointestinal cancer (GIC) screening[1-3]. Following the introduction of the CEA and CA19-9 assessment, in 1990, blood CA72-4 was proposed as a diagnostic biomarker for gastric cancer (GC)[4]. Subsequent studies showed that CA72-4 could be used to diagnose GC and colorectal cancer (CRC)[5,6]. These studies reported sensitivities of 19%-47% in GC and 25%-43% in CRC at the cut-off value of 6 kU/L[7-13]. The clinical guidelines published by the European Group on Tumor Markers (EGTM) in 2003 suggested that CA72-4 could be a potential biomarker for CRC[14].

Based on these previous studies, blood CA72-4 began to be widely used as a tumor biomarker since 2010 in China. Nevertheless, after large-scale clinical application, we noticed, empirically, an extremely high false positive rate of CA72-4 for diagnosis. A positive result would lead the subject to undergo further examinations, including gastroscopy, colonoscopy, chest computed tomography (CT), abdominal CT, and even positron emission tomography-CT (PET-CT). The blood test with a low positive predictive value (PPV) not only brings unnecessary anxiety, invasive examinations, and extra costs to the subjects but also leads to the waste of medical resources and increases the social medical burden.

The massive data and real-world diagnostic cohorts make it possible to further explore the diagnostic and economic value of biomarkers. Through a real-world diagnostic cohort, we comprehensively analyzed the differences in the levels of CEA, CA19-9, and CA72-4 and their diagnostic and economic value in gastrointestinal tumors. Four indicators were used to comprehensively evaluate the economic value, namely, the total cost and the average cost per person for each positive patient diagnosed and their corresponding cost-effectiveness ratios. We evaluated whether age and health checkup could help us make useful recommendations for thresholds of tumor biomarkers and medical insurance policies.

Table 1 The six schemes and examination prices in economic analysis

Item	Description
Schemes	Scheme 1 Gastrointestinal endoscopy for all people without blood tests
	Scheme 2 Both of gastroscopy and colonoscopy when blood tests were positive
	Scheme 3 Gastroscopy first when blood tests were positive, and then colonoscopy when the result of gastroscopy was negative
	Scheme 4 Colonoscopy first when blood tests were positive, and then gastroscopy when the result of colonoscopy was negative
	Scheme 5 Only gastroscopy when blood tests were positive
	Scheme 6 Only colonoscopy when blood tests were positive
Examination prices	CEA, $4.64; CA19-9, $7.25; CA72-4, $7.25
	Gastroscopy & biopsy, $87.99
	Colonoscopy, $57.98; biopsy after colonoscopy, $32.62

The prices of gastroscopy and colonoscopy did not include that of intravenous anesthesia. The costs of all examinations were from Zhongshan Hospital in 2019. All costs were converted to United States dollars. CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; CA72-4: Carbohydrate antigen 72-4.

MATERIALS AND METHODS

Study population

We retrospectively analyzed all patients from October 2006 to May 2018. The inclusion criteria included: (1) Patients from the medical examination center, outpatient department or inpatient department of Zhongshan Hospital of Fudan University; and (2) patients had completed all five examinations, namely, CEA, CA19-9, CA72-4, gastroscopy and colonoscopy, within half a year. The exclusion criteria were as follows: (1) Duplicate patients; and (2) patients who had accepted anti-tumor therapies such as radiotherapy, chemotherapy or surgery.

Data extraction

All data were abstracted from our hospital information system (HIS). They included general information (*e.g.*, age, sex, medical record number, whether health checkup, past history), the concentrations of each of the three tumor biomarkers, reports of auxiliary examinations (*e.g.*, endoscopy, pathology, ultrasonography, CT, magnetic resonance, PET-CT, electrocardiogram), and the medical records of outpatients and inpatients. The generation time of these data was also provided.

The concentrations of serum CEA, CA19-9, and CA72-4 were measured with an electrochemiluminescence immunoassay (Elecsys2010, Roche Diagnostics, indianapolis, IN, United States). The traditional cut-off values for CEA, CA19-9, and CA72-4 were 5 μg/L, 37 kU/L, and 6 kU/L, respectively.

According to the regular practice of our hospital, pathological biopsy was taken when gastroscopy was performed, while colon biopsy was not necessary taken unless some lesions were found by colonoscopy. The diagnosis of GIC depends on the gold standard of pathology, and other gastrointestinal diseases are diagnosed by endoscopy and pathology. Other malignant tumors were comprehensively judged based on the medical history, pathology and imaging exams that we could collect. TNM staging of cancers was based on the American Joint Committee on Cancer Staging or case data at that time.

Economic analysis

According to the type of test and the order of endoscopy procedures, we assumed six schemes (Table 1). Four economic indicators combined with the proportion of endoscopies and the missed diagnosis rate was used to evaluate the economic value of tumor biomarkers. The four economic indicators were the total cost and cost per capita of correctly diagnosing one case of GIC and the cost-effectiveness ratio of the above two indicators. The cost-effectiveness ratio was the total cost or cost per capita divided by sensitivity. We assumed that the missed diagnosis rate and misdiagnosis of endoscopy plus necessary pathological examination for gastrointestinal malignancies were all 0.

The costs of blood tests, endoscopy and pathological examination were the cost of these procedures at Zhongshan Hospital in 2019 (Table 1). All costs were converted to United States dollars.

Considering the preliminary results, further analyses were performed on Scheme 1 (gastrointestinal endoscopy for all people without blood tests) and Scheme 2 (both gastroscopy and colonoscopy when blood tests were positive). We also calculated 9 conditions when CEA and CA19-9 were combined. They

Table 2 The clinical characteristics of subjects with and without gastric cancer, colorectal cancer and gastrointestinal cancers

	Age median (quartile)	*P* value	Male, *n* (%)	Female, *n* (%)	*P* value
Gastric cancer	61 (51, 68)	< 0.001	268 (68.4)	124 (31.6)	0.084
Non-gastric cancer	48 (42, 56)		20831 (64.2)	11634 (35.8)	
Colorectal cancer	62 (55, 70)	< 0.001	522 (58.5)	370 (41.5)	< 0.001
Non-colorectal cancer	48 (42, 55)		20577 (64.4)	11388 (35.6)	
Gastrointestinal cancer	62 (53, 69)	< 0.001	816 (62.4)	491 (37.6)	0.170
Non-gastrointestinal cancer	48 (42, 55)		20283 (64.3)	11267 (35.7)	

The *P* value was calculated with the Wilcoxon test for age and chi-square test for sex. The bold font indicates that the *P* value was less than 0.05.

were parallel (any positive was considered positive), serial (all positive was considered positive), and the formula under the traditional cut-off value (the coefficients of CEA and CA19-9 were calculated according to the logistic regression), the minimum total cost, and the minimum total cost-effectiveness ratio.

Subgroup analysis (age, health checkup/active consultation) was utilized to analyze the economic value of the three biomarkers under the traditional threshold, with a view to drawing some medical insurance recommendations.

Statistical analysis

The statistical analyses were performed using R software 3.3.5 (R Foundation for Statistical Computing, Vienna, Austria). The level of significance was set at $P < 0.05$. All tests were two-sided.

Student's *t*-test or Wilcoxon test was used to assess the differences in continuous variables, as appropriate. The chi-square test was used for counting variables. Correlations between two variables were calculated by Pearson correlation analysis or Spearman correlation analysis. The influences of age and sex on the biomarker levels were analyzed with the regression coefficient of linear regression. Categorical regression analysis was utilized to calculate the regression coefficient quantification of each stage of GC and CRC.

The diagnostic value was evaluated by means of the area under the curve (AUC) values of the receiver operating characteristics (ROC) curve, as well as the diagnostic odds ratio (DOR), sensitivity, specificity, Youden index (sensitivity + specificity-1), accuracy, predicted value and likelihood ratio on the traditional and best cut-off values. The best cut-off value referred to the threshold when the Youden index was the largest. When multiple diagnostic biomarkers were combined, logistic regression was used to calculate the formula coefficients. We used Delong's test to compare AUC.

Decision curve analysis (DCA) was performed to determine the clinical usefulness of the radiomics nomogram by quantifying the net benefits at different threshold probabilities. The clinical net benefit was defined as the true positive rate (sensitivity) minus the false positive rate (misdiagnosis rate) and was then weighted by the relative damage of the positive rate and the negative rate.

RESULTS

Clinical characteristics

According to the inclusion criteria, we screened a total of 32857 subjects aged 15 to 97 years in the HIS, including 21099 males and 11758 females. There were 24045 subjects who underwent health checkup and 8812 subjects with an active consultation (Figure 1). The ages and sexes of the subjects with GC, CRC, and GIC were significantly different from those of the subjects without the disease (Table 2).

The constituent ratios of the diseases detected by gastroscopy, colonoscopy and pathological examination are displayed in Table 3. Among them, there were 392 GC cases, 892 CRC cases and 1307 GIC cases.

Serum levels of tumor biomarkers

The concentrations of the three biomarkers were skewed (Figure 2). The correlations between the pairwise biomarkers are shown in Table 4. We found that there were significant correlations between CEA and CA19-9 in all subjects and various GICs, and the correlation coefficients were all exceed 0.245.

The median values for CEA, CA19-9, and CA72-4 Level were 1.67 μg/L, 8.50 kU/L and 1.60 kU/L, respectively. The expression levels of the biomarkers for the diseases that had more than 30 cases are shown in Table 5. The concentrations of the three biomarkers in patients with several malignant tumors were significantly different from those without malignant tumors (Figure 3). The CEA level increased

Table 3 The number and proportions of diseases in the gastroscopy, colonoscopy and pathological examination groups

Examination	Disease	No.	%
Gastroscope (without pathological examination)	Esophagitis	2887	8.8%
	Esophageal erosion	88	0.3%
	Esophageal ulcer	30	0.1%
	Esophageal protuberant lesion	491	1.5%
	Esophageal non-protuberant lesion	77	0.2%
	Barrett's esophagus	66	0.2%
	Bile reflux	1344	4.1%
	Gastric atrophy	45	0.1%
	Gastric erosion	12457	37.9%
	Gastric hemorrhage	1117	3.4%
	Gastric ulcer	1088	3.3%
	Gastric protuberant lesion	3329	10.1%
	Gastric non-protuberant lesion	225	0.7%
	Duodenitis	1473	4.5%
	Duodenal erosion	26	0.1%
	Duodenal ulcer	1548	4.7%
	Duodenal protuberant lesion	666	2.0%
	Duodenal non-protuberant lesion	31	0.1%
Colonoscopy (without pathological examination)	Colorectitis	653	2.0%
	Colorectal erosion	29	0.1%
	Colorectal ulcer	99	0.3%
	Colorectal protuberant lesion	7312	22.3%
	Colorectal non-protuberant lesion	36	0.1%
Pathological examination	Esophageal mucositis	398	1.2%
	Esophageal dysplasia	44	0.1%
	Esophageal adenoma	1	< 0.1%
	Esophageal hyperplastic polyp	2	< 0.1%
	Esophageal glandular hyperplasia	4	< 0.1%
	Chronic atrophic gastritis	1809	5.5%
	Gastric dysplasia	308	0.9%
	Gastric adenoma	13	< 0.1%
	Gastric hyperplastic polyps	117	0.4%
	Gastric glandular hyperplasia	761	2.3%
	Gastric juvenile polyps	1	< 0.1%
	Duodenal mucositis	276	0.8%
	Duodenal dysplasia	24	0.1%
	Duodenal adenoma	12	< 0.1%
	Duodenal hyperplastic polyps	10	< 0.1%
	Duodenal gland hyperplasia	31	0.1%
	Colorectal mucositis	2206	6.7%
	Colorectal high-grade intraepithelial neoplasia	330	1.0%

	Colorectal low-grade intraepithelial neoplasia	3364	10.2%
	Colorectal adenoma	3707	11.3%
	Colorectal hyperplastic polyps	1037	3.2%
	Colorectal inflammatory polyps	13	< 0.1%
	Colorectal gland hyperplasia	567	1.7%
	Colorectal juvenile polyps	3	< 0.1%
	Peutz-Jeghers polyps	5	< 0.1%
	Familial polyposis coli	3	< 0.1%
	Esophageal cancer	57	0.2%
	Gastric cancer	392	1.2%
	Duodenal cancer	25	0.1%
	Small intestine cancer	4	< 0.1%
	Colorectal cancer	892	2.7%
	Liver cancer	127	0.4%
	Pancreatic cancer	47	0.1%
	Gallbladder cancer	20	0.1%
	Bile duct cancer & ampulla cancer	10	< 0.1%
	Lung cancer	129	0.4%
	Breast cancer	55	0.2%
	Ovarian cancer	57	0.2%
	Uterine malignancy	28	0.1%
	Kidney cancer	37	0.1%
	Prostate cancer	28	0.1%
	Bladder Cancer	17	0.1%
	Leukemia	1	< 0.1%
	Lymphoma	29	0.1%
	Other malignant tumors	15	< 0.1%

Table 4 Correlation analysis of biomarker levels

	Correlation coefficient			*P* value		
	CEA and CA19-9	CEA and CA72-4	CA19-9 and CA724-4	CEA and CA19-9	CEA and CA72-4	CA19-9 and CA724-4
Whole	0.245	-0.005	-0.046	< 0.001	0.359	< 0.001
Gastric cancer	0.291	0.048	0.022	< 0.001	0.342	0.657
Colorectal cancer	0.385	0.2	0.169	< 0.001	< 0.001	< 0.001
Gastrointestinal cancer	0.354	0.164	0.134	< 0.001	< 0.001	< 0.001

P values and correlation coefficients were calculated with Pearson correlation analysis or Spearman correlation analysis. The bold font indicates that the *P* value was less than 0.05. CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; CA72-4: Carbohydrate antigen 72-4.

but did not exceed 0.3 μg/L in esophageal erosion, gastric erosion, gastric ulcer, chronic atrophic gastritis, and colorectal adenoma.

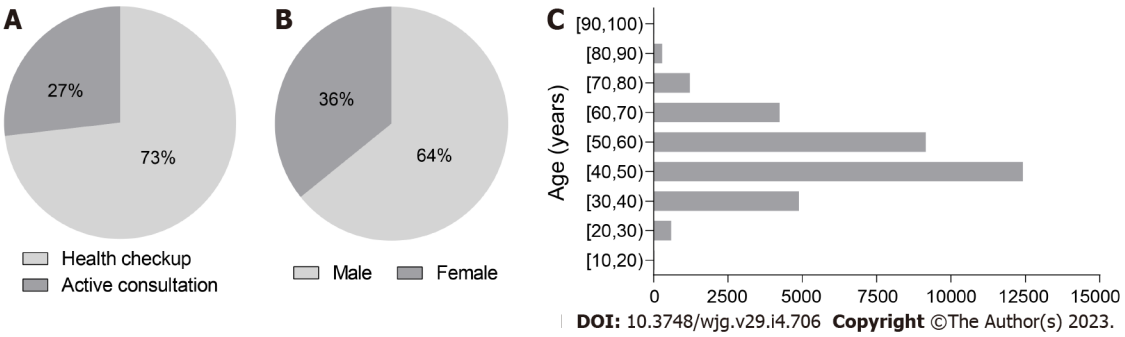
The influences of age and sex on the biomarker levels are presented in Table 6. Due to the fact that the patients with malignant tumors were elder, the age baselines of the patients with and without tumors were no equal. Moreover, the sex baseline of the CRC patients was not the same. The correlation coefficients of age and sex were both less than 0.25, indicating small influences. The regression coefficients

Table 5 The biomarker levels, comparisons between subjects with and without diseases and area under the curves of carcinoembryonic antigen, carbohydrate antigen 19-9, and carbohydrate antigen 72-4

Disease	No.	CEA			CA19-9			CA72-4		
		Median (quartile) (μg/L)	*P* value	AUC	Median (quartile) (kU/L)	*P* value	AUC	Median (quartile) (kU/L)	*P* value	AUC
Whole	32857	1.67 (1.13, 2.41)	-	-	8.50 (5.60, 13.50)	-	-	1.60 (1.05, 3.20)	-	-
Esophagitis	3137	1.88 (1.29, 2.72)	< 0.001	0.566	8.60 (5.70, 13.50)	0.439	0.504	1.60 (1.10, 3.40)	0.001	0.518
Esophageal erosion	109	1.84 (1.40, 2.90)	0.007	0.575	8.30 (5.00, 15.00)	0.938	0.498	1.50 (1.10, 3.20)	0.644	0.487
Esophageal ulcer	30	1.73 (0.90, 2.29)	0.818	0.488	7.90 (5.73, 10.38)	0.166	0.573	1.59 (1.10, 3.95)	0.572	0.470
Barrett's esophagus	66	1.91 (1.21, 3.01)	0.095	0.559	8.95 (6.25, 12.70)	0.565	0.520	1.95 (1.10, 3.98)	0.306	0.536
Bile reflux	1344	1.65 (1.07, 2.43)	0.435	0.506	8.90 (5.60, 14.73)	0.121	0.512	1.60 (1.10, 3.30)	0.130	0.512
Gastric erosion	13094	1.78 (1.22, 2.57)	< 0.001	0.555	8.60 (5.70, 13.70)	0.006	0.509	1.60 (1.00, 3.20)	0.748	0.499
Gastric ulcer	1091	1.98 (1.41, 2.98)	< 0.001	0.598	8.40 (5.50, 14.40)	0.646	0.496	1.60 (1.00, 3.20)	0.789	0.498
Gastric hemorrhage	1125	1.68 (1.12, 2.46)	0.492	0.506	8.70 (5.80, 14.50)	0.092	0.515	1.60 (1.10, 3.40)	0.331	0.509
Chronic atrophic gastritis	1839	1.90 (1.32, 2.89)	< 0.001	0.578	9.20 (6.00, 14.90)	< 0.001	0.535	1.70 (1.10, 3.40)	< 0.001	0.528
Gastric xanthoma	100	1.65 (1.13, 2.41)	0.935	0.498	10.35 (6.45, 15.18)	0.072	0.552	1.80 (1.20, 3.43)	0.111	0.546
Gastrointestinal stromal tumor	48	1.56 (1.09, 2.69)	0.903	0.505	7.80 (5.78, 13.41)	0.614	0.521	1.50 (1.10, 2.23)	0.683	0.517
Gastric hyperplastic polyps	117	1.71 (1.14, 2.89)	0.282	0.529	11.20 (7.00, 22.72)	< 0.001	0.612	1.70 (1.10, 2.40)	0.964	0.501
Gastric glandular hyperplasia	761	1.57 (1.09, 2.29)	0.050	0.521	9.50 (6.00, 15.00)	< 0.001	0.543	1.70 (1.10, 3.80)	0.010	0.527
Colorectitis	2592	1.83 (1.25, 2.70)	< 0.001	0.552	8.70 (5.70, 14.30)	0.014	0.515	1.60 (1.10, 3.40)	0.034	0.513
Colorectal erosion	167	1.72 (1.17, 2.73)	0.198	0.529	8.80 (5.95, 13.90)	0.495	0.515	1.60 (1.00, 3.10)	0.788	0.494
Colorectal ulcer	107	1.54 (1.03, 2.61)	0.658	0.512	9.10 (6.30, 16.90)	0.069	0.551	1.70 (1.09, 2.80)	0.859	0.495
Colorectal hemorrhage	36	1.57 (1.12, 3.14)	0.799	0.488	9.05 (5.73, 13.75)	0.550	0.529	1.25 (0.90, 1.95)	0.061	0.590
Colorectal cyst	41	1.53 (0.93, 2.70)	0.455	0.534	9.20 (6.50, 13.70)	0.321	0.545	1.40 (1.10, 2.40)	0.353	0.542
Colorectal adenoma	3707	1.91 (1.29, 2.84)	< 0.001	0.578	9.04 (6.00, 14.70)	< 0.001	0.532	1.60 (1.10, 3.30)	0.010	0.513
Colorectal hyperplastic polyps	1037	1.88 (1.31, 2.75)	< 0.001	0.565	8.70 (5.90, 13.70)	0.065	0.517	1.60 (1.00, 3.20)	0.379	0.492
Colorectal gland hyperplasia	567	1.87 (1.35, 2.62)	< 0.001	0.560	8.30 (5.45, 13.45)	0.308	0.512	1.50 (1.10, 3.30)	0.687	0.505
Esophageal cancer	57	2.34 (1.30, 3.78)	< 0.001	0.645	9.40 (6.40, 20.00)	0.114	0.560	2.00 (1.20, 4.20)	0.073	0.568
Gastric cancer	392	2.15 (1.35, 4.13)	< 0.001	0.625	10.30 (5.70, 20.23)	< 0.001	0.577	2.00 (1.10, 5.70)	< 0.001	0.570
Colorectal cancer	892	3.25 (1.78, 11.55)	< 0.001	0.736	13.30 (7.10, 33.45)	< 0.001	0.649	2.30 (1.20, 5.90)	< 0.001	0.598
Liver cancer	127	4.27 (2.15, 7.46)	< 0.001	0.786	17.30 (7.40, 39.15)	< 0.001	0.674	1.60 (1.15, 3.75)	0.070	0.547
Pancreatic cancer	47	3.20 (1.98, 9.63)	< 0.001	0.771	99.60 (16.95, 307.15)	< 0.001	0.830	3.10 (1.35, 9.60)	< 0.001	0.680
Lung cancer	129	4.25 (2.15, 16.67)	< 0.001	0.787	12.10 (8.20, 25.50)	< 0.001	0.668	3.40 (1.40, 9.00)	< 0.001	0.660
Breast cancer	55	2.39 (1.44, 6.06)	< 0.001	0.654	15.60 (8.30, 27.20)	< 0.001	0.693	2.10 (1.20, 4.70)	0.022	0.589
Ovarian cancer	57	1.78 (1.08, 5.63)	0.228	0.546	21.85 (8.50, 130.50)	< 0.001	0.718	6.40 (1.50, 17.70)	< 0.001	0.698

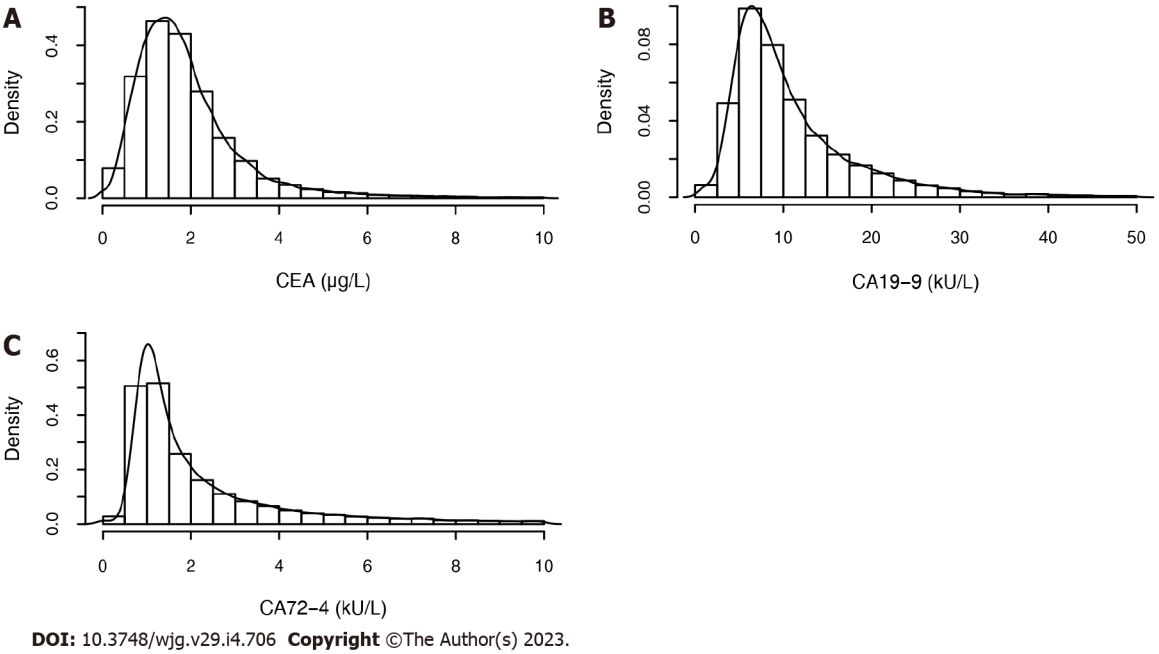
Thyroid cancer	74	1.71 (1.06, 2.48)	0.936	0.497	9.90 (6.80, 15.28)	0.067	0.562	1.70 (1.03, 3.08)	0.874	0.495
Kidney cancer	37	2.37 (1.16, 3.70)	0.015	0.615	10.60 (7.70, 17.46)	0.039	0.598	2.20 (1.40, 5.20)	0.050	0.593
Malignant tumors (except thyroid cancer)	1955	2.65 (1.49, 6.70)	< 0.001	0.692	12.00 (6.80, 28.30)	< 0.001	0.636	2.20 (1.20, 5.90)	< 0.001	0.589

The bold font of the *P* value indicates that the *P* value was less than 0.05 in the Wilcoxon test. The bold font of the area under the curve (AUC) indicates that the AUC value was greater than 0.7. CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; CA72-4: Carbohydrate antigen 72-4; AUC: Area under the curve.



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Figure 1 Fan charts and bar plots of the clinical characteristic. A-C: There were 24045 subjects who underwent health checkup and 8812 subjects with an active consultation.



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Figure 2 Histograms of carcinoembryonic antigen, carbohydrate antigen 19-9, and carbohydrate antigen 72-4. A: Carcinoembryonic antigen; B: Carbohydrate antigen 19-9; C: Carbohydrate antigen 72-4. CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9; CA 72-4: Carbohydrate antigen 72-4.

were used to calculate the effect of age on the biomarker levels. CEA, CA19-9, and CA72-4 increased by 0.41, 2.69, and 0.69, respectively, for the subjects without malignant tumors for every 10-year increase.

The biomarker levels in different malignant tumor stages are shown in Table 7 and Figure 4.

Diagnostic accuracies of tumor biomarkers

The AUCs of the three biomarkers in various benign and malignant diseases are displayed in Table 5, and the ROC curves are shown in Figure 5. An AUC above 0.7 was of moderate diagnostic value, and an AUC above 0.9 was of high diagnostic value. We found that even though the biomarker levels of several

Table 6 Correlation analysis and linear regression analysis of biomarker levels and clinical characteristics

		Age			Gender		
		***P* value**	**Correlation coefficient**	**Regression coefficient**	***P* value**	**Correlation coefficient**	**Regression coefficient**
CEA	Whole	< 0.001	0.227	0.176	< 0.001	0.236	0.004
	With malignant tumors	< 0.001	0.231	0.263	< 0.001	0.144	-2.965
	Without malignant tumors	< 0.001	0.195	0.041	< 0.001	0.248	0.472
CA19-9	Whole	< 0.001	0.135	1.076	< 0.001	-0.070	-1.400
	With malignant tumors	< 0.001	0.111	1.898	0.356	-0.021	-
	Without malignant tumors	< 0.001	0.113	0.269	< 0.001	-0.071	-2.482
CA72-4	Whole	< 0.001	0.084	0.076	< 0.001	-0.043	-0.927
	With malignant tumors	0.064	0.042	-	0.814	-0.005	-
	Without malignant tumors	< 0.001	0.069	0.052	< 0.001	-0.044	-0.773

The bold font indicates that the *P* value was less than 0.05. CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; CA72-4: Carbohydrate antigen 72-4.

diseases were significantly different, the diagnostic values of these biomarkers were not high enough. The AUC of the CEA level reached 0.7 for CRC, liver cancer, pancreatic cancer and lung cancer, while those of the CA19-9 Level reached 0.830 for pancreatic cancer and 0.7 for ovarian cancer. There was no disease in which the AUC of CA72-4 reached 0.7.

We show the diagnostic value of GC, CRC and gastrointestinal malignant tumors (the DOR, sensitivity, specificity, Youden index, accuracy, predictive value, likelihood ratio under the traditional and the best threshold) in Table 8. Furthermore, we provide several criteria for evaluating their diagnostic efficiencies as the qualified standards: positive likelihood ratio, negative likelihood ratio and DOR should be > 5.0, < 0.2 and > 10.0, respectively. Generally, there is no ideal biomarker for GC. In this study, CEA was better than CA19-9 and CA72-4. The positive likelihood ratio and DOR of CEA and CA19-9 were qualified for CRC and GIC, while those of CA72-4 were not qualified for GC, CRC or GIC.

The AUCs of diverse subgroups, including age, health checkup/active consultation and malignant tumor stage, are shown in Table 9. We defined an AUC greater than 0.7 as the qualified line. Then, the AUCs of CEA, CA19-9, and CA72-4 in the health checkup population were all unqualified. If we looked at the stages alone, CEA for stage-IV GC, CA19-9 for stage-IV CRC and CEA for stage-II-IV CRC were qualified. However, neither CEA nor CA199 can diagnose early GICs.

The DCA curves of the three biomarkers are presented in Figure 6. The DCA curve showed that under the traditional threshold and the best threshold, the clinical benefits of CEA were higher than those of CA19-9, while the clinical benefits of CA72-4 were the lowest.

Four panels were conducted with the combination of the three biomarkers. We selected the panel with the highest AUC and compared it with the single biomarker with the highest AUC (Table 10). The combination of biomarkers in the CRC and gastrointestinal malignant tumors significantly increased the AUC (Delong's test, P < 0.05) by less than 0.3, while that in GC did not. Therefore, the combination of the three biomarkers could not greatly improve the diagnostic value.

Economic analysis of tumor biomarkers with endoscopies

We analyzed the four economic indicators of the six schemes with changes in the serum levels of the three biomarkers, as shown in Figure 7. For gastroscopy only, the total cost and cost-effectiveness ratio of correctly diagnosing one case of GIC were unacceptably high. For colonoscopy only, various cost indicators were reduced within a certain range of biomarker levels. The four economic indicators of CEA in Scheme 6 (only colonoscopy conducted when blood tests were positive) were lower than those in other schemes because the diagnostic efficiencies of CEA for CRC were high, and the prevalence rate of CRC was higher than that of GC in this study. If both gastroscopy and colonoscopy were conducted, the influence of the order of gastroscopy on the four economic indicators was small. Therefore, in the follow-up study, we only calculated the economic indicators in Scheme 2 (both gastroscopy and colonoscopy when blood tests were positive) compared to those in Scheme 1 (both gastroscopy and colonoscopy for all people without blood tests).

Table 7 The biomarker levels and categorical regression analysis of each stage of gastric cancer and colorectal cancer

		Gastric cancer		Colorectal cancer	
		Median (quartile)	Quatization	Median (quartile)	Quatization
CEA (μg/L)	CIS	1.74 (1.45, 2.18)	-0.983	2.04 (1.17, 2.32)	-1.695
	Stage I	1.78 (1.29, 2.79)	-0.983	2.30 (1.45, 4.10)	-1.252
	Stage II	2.16 (1.07, 4.03)	-0.835	3.42 (2.13, 9.26)	-0.680
	Stage III	2.37 (1.31, 5.78)	-0.176	3.28 (2.00, 8.21)	-0.680
	Stage IV	3.79 (1.76, 29.1)	1.346	10.1 (2.57, 57.4)	1.168
CA19-9 (kU/L)	CIS	8.60 (5.05, 12.7)	-1.138	8.66 (6.18, 13.2)	-0.963
	Stage I	9.25 (5.88, 14.4)	-1.138	9.50 (6.20, 14.1)	-0.963
	Stage II	7.73 (5.53, 16.8)	-1.138	12.4 (7.38, 28.8)	-0.812
	Stage III	12.2 (5.63, 37.7)	0.842	13.1 (7.33, 23.0)	-0.790
	Stage IV	11.8 (5.08, 28.7)	0.903	28.9 (10.5, 216.4)	1.192
CA72-4 (kU/L)	CIS	1.50 (0.90, 3.50)	-1.203	1.50 (1.00, 2.03)	-1.550
	Stage I	1.80 (1.20, 4.33)	-0.977	1.70 (1.10, 3.20)	-1.060
	Stage II	1.90 (1.18, 4.30)	-0.789	2.10 (1.20, 3.81)	-0.818
	Stage III	2.10 (1.30, 4.63)	-0.164	1.95 (1.10, 4.21)	-0.679
	Stage IV	4.10 (1.00, 12.0)	1.340	4.75 (1.50, 15.2)	1.182

CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; CA72-4: Carbohydrate antigen 72-4; CIS: Carcinoma *in situ*.

Table 8 Diagnostic efficiencies of gastric cancer, colorectal cancer and gastrointestinal cancers at the traditional and best cut-off values

		Gastric cancer			Colorectal cancer			Gastrointestinal cancers		
		CEA	CA19-9	CA72-4	CEA	CA19-9	CA72-4	CEA	CA19-9	CA72-4
	AUC	0.625	0.577	0.570	0.736	0.649	0.598	0.705	0.627	0.590
Traditional cut-off value	Cut-off value	5.0	37.0	6.0	5.0	37.0	6.0	5.0	37.0	6.0
	DOR	6.083	5.089	2.220	14.854	11.895	2.376	12.459	10.367	2.337
	Sensitivity	0.227	0.140	0.240	0.377	0.241	0.249	0.323	0.210	0.243
	Specificity	0.954	0.969	0.876	0.961	0.974	0.878	0.963	0.975	0.879
	Youden index	0.181	0.109	0.115	0.338	0.215	0.127	0.286	0.185	0.122
	Accuracy	0.945	0.959	0.868	0.945	0.954	0.861	0.938	0.945	0.854
	PPV	0.056	0.052	0.023	0.212	0.204	0.054	0.266	0.261	0.077
	NPV	0.990	0.989	0.990	0.982	0.979	0.977	0.972	0.968	0.966
	PLR	4.927	4.516	1.927	9.640	9.269	2.034	8.759	8.400	2.012
	NLR	0.810	0.888	0.868	0.649	0.779	0.856	0.703	0.810	0.861
Best cut-off value	Cut-off value	2.6	16.3	3.8	2.8	20.7	2.0	2.5	19.6	3.4
	DOR	2.687	2.233	2.038	6.345	4.825	1.933	4.419	3.872	2.068
	Sensitivity	0.423	0.324	0.349	0.558	0.361	0.566	0.556	0.339	0.375
	Specificity	0.785	0.823	0.791	0.834	0.895	0.597	0.779	0.883	0.775
	Youden index	0.209	0.147	0.141	0.392	0.256	0.163	0.335	0.222	0.150
	Accuracy	0.781	0.817	0.786	0.827	0.881	0.596	0.770	0.861	0.759
	PPV	0.023	0.022	0.020	0.086	0.088	0.038	0.094	0.107	0.065

	NPV	0.991	0.990	0.990	0.985	0.980	0.980	0.977	0.970	0.968
	PLR	1.972	1.833	1.675	3.363	3.445	1.405	2.519	2.900	1.667
	NLR	0.734	0.821	0.822	0.530	0.714	0.727	0.570	0.749	0.806

The best cut-off value referred to the threshold when the Youden index was the largest. CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; CA72-4: Carbohydrate antigen 72-4; AUC: Area under the curve; DOR: Diagnostic odds ratio; PPV: Positive predictive value; NPV: Negative predictive value; PLR: Positive likelihood ratio; NLR: Negative likelihood ratio.

Table 9 Subgroup analysis of area under the curve for gastric cancer, colorectal cancer and gastrointestinal cancers

	Gastric cancer			Colorectal cancer			Gastrointestinal cancer		
	CEA	CA19-9	CA72-4	CEA	CA19-9	CA72-4	CEA	CA19-9	CA72-4
Whole	0.625	0.577	0.570	0.736	0.649	0.598	0.705	0.627	0.590
≥ 60 years	0.585	0.521	0.544	0.701	0.614	0.577	0.675	0.592	0.572
< 60 years	0.578	0.571	0.570	0.683	0.616	0.593	0.648	0.598	0.583
HC	0.570	0.570	0.525	0.584	0.539	0.514	0.584	0.554	0.526
AC	0.595	0.544	0.547	0.724	0.637	0.577	0.696	0.615	0.571
CIS	0.551	0.478	0.542	0.540	0.536	0.526	-	-	-
Stage I	0.554	0.525	0.565	0.675	0.546	0.512	-	-	-
Stage II	0.603	0.489	0.591	0.781	0.657	0.578	-	-	-
Stage III	0.658	0.645	0.603	0.770	0.642	0.565	-	-	-
Stage IV	0.739	0.614	0.634	0.810	0.778	0.698	-	-	-

The bold font indicates that the area under the curve value was more than 0.7. CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; CA72-4: Carbohydrate antigen 72-4; HC: Health checkup; AC: Active consultation; CIS: Carcinoma *in situ*.

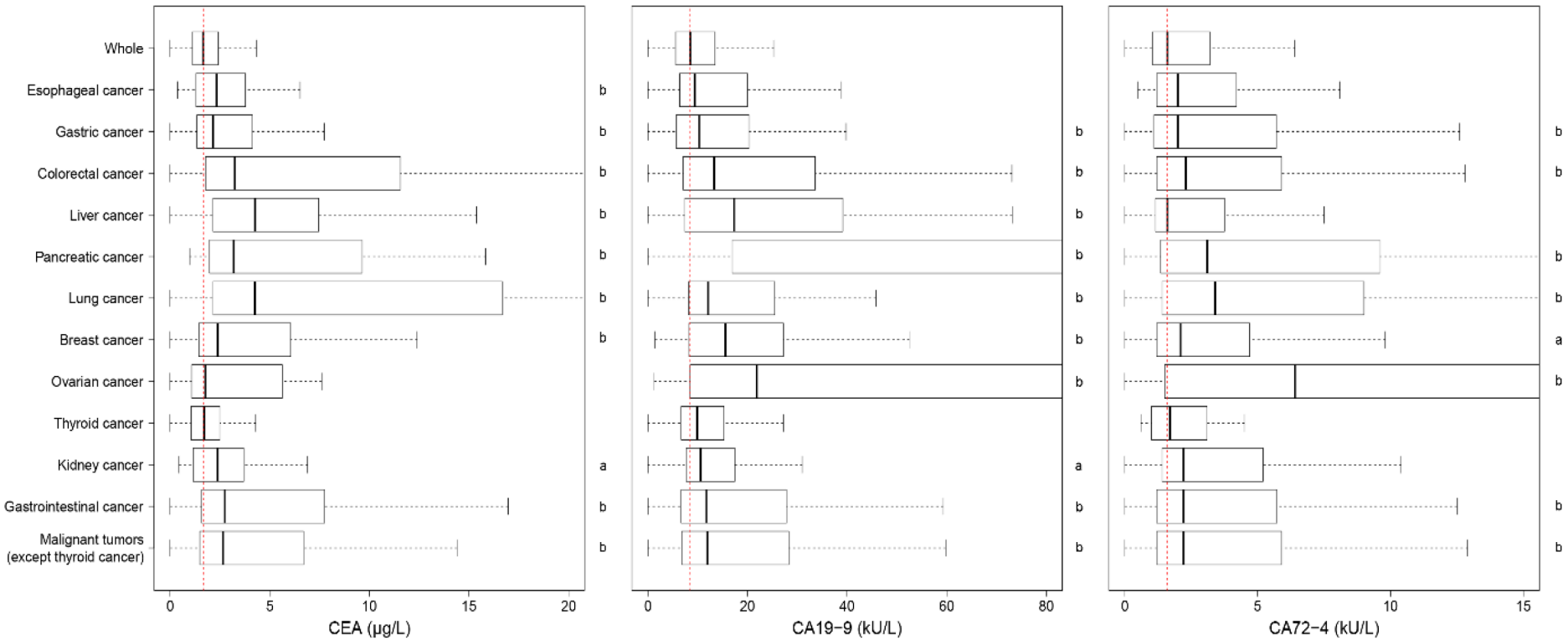
Table 10 The best single biomarker and the best combination of biomarkers for gastric cancer, colorectal cancer and gastrointestinal cancers

	Best combination		Best single biomarker		*P* value
	Biomarkers	AUC	Biomarker	AUC	
Gastric cancer	CEA + CA19-9 + CA72-4	0.653	CEA	0.625	0.067
Colorectal cancer	CEA + CA19-9	0.761	CEA	0.736	< 0.001
Gastrointestinal cancers	CEA + CA19-9	0.727	CEA	0.705	< 0.001

The bold font indicates that the *P* value was less than 0.05 in Delong's test. AUC: Area under the curve; CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; CA72-4: Carbohydrate antigen 72-4.

In terms of threshold selection, we found that the traditional threshold of CEA (5 μg/L) was exactly between the CEA level under the minimum total cost-effectiveness ratio (4.3 μg/L) and that under the minimum total cost (equal to cost-effectiveness ratio per capita, 8.7 μg/L). If we decrease the cut-off value, the four indicators grew rapidly. If we increase the cut-off value, then the total cost-effectiveness ratio rose sharply, while the other three indicators had fewer changes. One can use 5 μg/L for CEA as an economic cut-off value. For CA19-9, we found that a similarly high economic cut-off value was approximately 30 kU/L, not the traditional threshold of 37 kU/L. Compared with that at the threshold of 30 kU/L, the total cost-effectiveness ratio at the threshold of 37 kU/L was greatly increased because of the lower sensitivity of the marker. We evaluated the economic efficiencies as the qualified standard: all four indicators in Scheme 2 were lower than those in Scheme 1. CEA met the standards at the threshold of 1.8 μg/L to 10.4 μg/L. CA19-9 and CA72-4 failed at the whole threshold, caused by the high total cost-effectiveness ratio in Scheme 2.

Compared with CEA, the combination of the three biomarkers in pairs or altogether caused the cost and cost-effectiveness ratio to be higher (Table 11). From an economic perspective, the combination of



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Figure 3 Boxplots of biomarker levels of malignant tumors. The red dotted line is the biomarker level for all subjects. The *P* value was calculated between the malignant tumor patients and the subjects without any malignant tumors by the Wilcoxon test. [a]*P* < 0.05, [b]*P* < 0.01. CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9; CA 72-4: Carbohydrate antigen 72-4.

biomarkers is not superior to the single biomarker, CEA.

Economic analysis of tumor biomarkers in different subgroups

The subgroup analysis under the traditional threshold is displayed in Table 12, Figures 8-10.

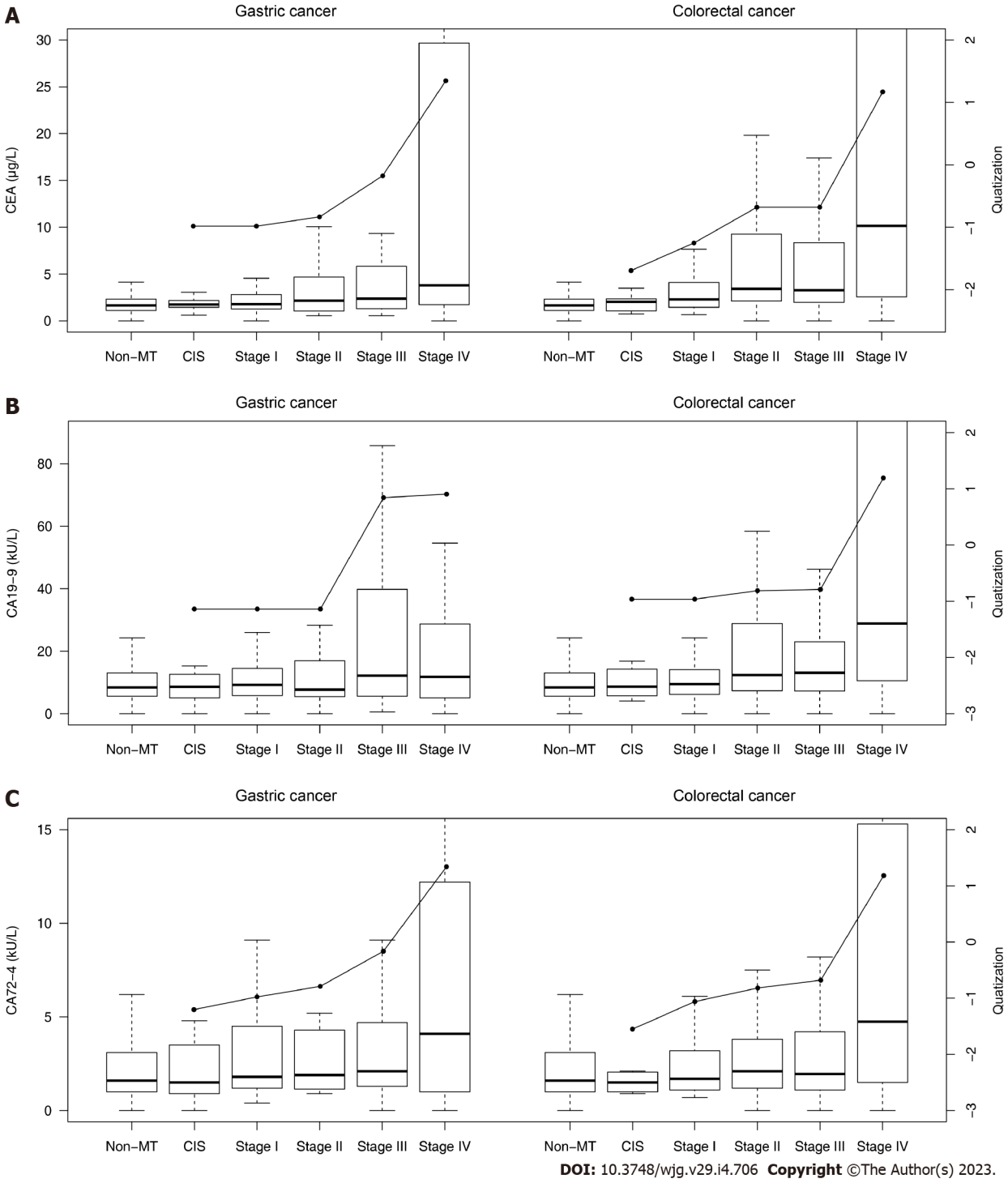
As we expected, for all ages, the four economic indicators of CEA in the health checkup subgroup were much higher than those in the active consultation subgroup. In the subgroup of health checkup subjects above 65 years old, all four indicators of CEA in Scheme 2 were lower than those in Scheme 1, while the total cost-effectiveness ratio in Scheme 2 was higher than that in Scheme 1 in the subgroup of health checkup subjects under 60 years. This highlights that conducting CEA testing in the health checkup for people over 65 years old is economically valuable, especially the lower cost per capita ($40.9 in Scheme 2 *vs* $146.6 in Scheme 1).

Table 11 Economic analysis of carcinoembryonic antigen, carbohydrate antigen 19-9, and carbohydrate antigen 72-4 in several situations for gastrointestinal cancers

	Cut-off value	Proportion of endoscopy	Missed diagnosis rate	Total cost ($)	Cost per capita ($)	Total C/E ($)	C/E per capita ($)	Remarks
Non-blood test	-	1.000	0.000	3574.3	146.9	3574.3	146.9	Gold standard
CEA (μg/L)	0.0	1.000	0.000	3691.9	151.7	3691.9	151.7	Lowest cut-off value
	2.5	0.230	0.451	1718.1	38.7	3130.1	70.6	Highest youden index
	4.3	0.065	0.642	990.0	14.6	2767.1	40.7	Lowest total cost-effectiveness ratio
	5.0	0.049	0.674	903.1	12.1	2770.9	37.1	Traditional diagnostic cut-off value
	8.7	0.020	0.759	783.9	7.8	3246.2	32.2	Lowest total cost & lowest cost-effectiveness ratio per capita
CA19-9 (kU/L)	0.0	1.000	0.000	3755.4	154.3	3755.4	154.3	Lowest cut-off value & lowest total cost-effectiveness ratio
	20.0	0.121	0.665	1836.7	25.3	5485.7	75.5	Highest youden index
	36.9	0.033	0.787	1398.5	12.2	6578.5	57.5	Lowest total cost & lowest cost-effectiveness ratio per capita
	37.0	0.032	0.789	1405.2	12.2	6656.1	57.7	Traditional diagnostic cut-off value
CA72-4 (kU/L)	0.0	1.000	0.000	3755.4	154.3	3755.4	154.3	Lowest cut-off value & lowest total cost-effectiveness ratio
	3.4	0.231	0.623	2670.7	41.4	7083.5	109.7	Highest youden index
	6.0	0.126	0.756	2584.5	25.9	10605.1	106.2	Traditional diagnostic cut-off value
	10.5	0.064	0.833	2451.6	16.8	14709.6	100.7	Lowest total cost & lowest cost-effectiveness ratio per capita
CEA	5.0	0.069	0.601	1365.1	22.4	3419.2	56.1	Traditional diagnostic cut-off value in parallel
CA19-9	37.0							
CEA	6.9	0.036	0.676	1309.0	17.4	4034.6	53.8	Lowest cut-off value & lowest total cost-effectiveness ratio in parallel
CA19-9	69.2							
CEA	3.9	0.098	0.554	1455.6	26.7	3264.3	59.8	Lowest total cost-effectiveness ratio in parallel
CA19-9	38.1							
CEA	5.0	0.012	0.862	2433.3	13.8	17661.0	100.0	Traditional diagnostic cut-off value in serial
CA19-9	37.0							
CEA	5.4	0.042	0.689	1437.7	18.4	4621.3	59.1	Lowest cut-off value & lowest total cost-effectiveness ratio in serial
CA19-9	0.0							
CEA	2.1	0.335	0.361	2339.4	61.4	3659.5	96.1	Lowest total cost-effectiveness ratio in serial
CA19-9	0.0							
CEA	5.0	0.044	0.666	1362.9	18.7	4079.6	56.0	Traditional diagnostic cut-off value in the logistic model
CA19-9	37.0							
CEA	4.9	0.052	0.641	1341.0	19.8	3732.7	55.1	Lowest cut-off value & lowest total cost-effectiveness ratio in the logistic model
CA19-9	23.2							
CEA	2.0	0.213	0.436	1874.8	43.5	3321.5	77.0	Lowest total cost-effect-

CA19-9	33.5	iveness ratio in the logistic model

The economic indicators were total cost, cost per capita and their cost-effectiveness ratios. The cut-off value with the lowest total cost equaled that of the lowest cost-effectiveness ratio per capita. In the parallel test, any positive result of carcinoembryonic antigen (CEA) or carbohydrate antigen 19-9 (CA 19-9) was considered positive, while both of the positive results of CEA and CA19-9 were considered positive in the serial test. The logistic model was CEA × 2.02 + CA19-9 × 0.06, which made the area under the curve highest. C/E: Cost-effectiveness ratio; CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; CA72-4: Carbohydrate antigen 72-4.



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Figure 4 Boxplots of biomarker levels for each stage of gastric cancer and colorectal cancer. A: Carcinoembryonic antigen; B: Carbohydrate antigen 19-9; C: Carbohydrate antigen 72-4. CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9; CA 72-4: Carbohydrate antigen 72-4; CIS: Carcinoma *in situ*.

Table 12 Subgroup analysis of economic indicators

			Scheme 2							Scheme 1	
			Cut-off value	Proportion of endoscopy	Missed diagnosis rate	Total cost ($)	Cost per capita ($)	Total C/E ($)	C/E per capita ($)	Total cost & C/E ($)	Cost & C/E per capita ($)
CEA	Whole	Whole	5.0	0.049	0.674	903.1	12.1	2770.9	37.1	3574.3	146.9
		≥ 80 yr	5.0	0.258	0.543	381.8	45.6	835.9	99.8	584.4	152.7
		≥ 75 yr	5.0	0.240	0.635	472.1	41.9	1294.7	115.0	623.2	151.8
		≥ 70 yr	5.0	0.214	0.627	463.5	37.7	1242.1	101.2	691.5	150.9
		≥ 65 yr	5.0	0.174	0.634	486.4	31.6	1329.1	86.3	844.6	149.9
		≥ 60 yr	5.0	0.137	0.636	523.6	25.8	1438.6	71.0	1099.1	149.0
		≥ 55 yr	5.0	0.110	0.639	574.1	21.6	1588.8	59.8	1424.3	148.3
		≥ 50 yr	5.0	0.085	0.643	662.6	17.7	1854.8	49.6	1972.8	147.6
		≥ 45 yr	5.0	0.065	0.658	758.5	14.6	2220.9	42.7	2614.6	147.2
		≥ 40 yr	5.0	0.055	0.668	837.4	13.1	2520.1	39.4	3121.4	147.0
		≥ 35 yr	5.0	0.051	0.671	882.1	12.4	2684.9	37.8	3427.4	146.9
		≥ 30 yr	5.0	0.049	0.674	902.2	12.2	2764.8	37.3	3552.0	146.9
	HC	Whole	5.0	0.021	0.883	6834.3	7.7	58218.5	65.4	15277.9	146.1
		≥ 80 yr	5.0	0.238	0.000	446.3	42.5	446.3	42.5	1565.3	149.1
		≥ 75 yr	5.0	0.129	0.500	941.8	24.4	1883.7	48.7	2843.9	147.1
		≥ 70 yr	5.0	0.101	0.538	693.8	20.0	1503.3	43.3	2356.2	146.9
		≥ 65 yr	5.0	0.070	0.630	1046.6	15.1	2832.1	40.9	3755.2	146.6
		≥ 60 yr	5.0	0.049	0.744	1677.6	12.0	6542.7	46.7	5254.5	146.4
		≥ 55 yr	5.0	0.040	0.794	2611.7	10.5	12685.3	51.1	7474.5	146.3
		≥ 50 yr	5.0	0.033	0.824	3519.3	9.5	19989.5	53.8	9558.2	146.2
		≥ 45 yr	5.0	0.026	0.848	4577.8	8.5	30179.8	55.7	12010.0	146.2
		≥ 40 yr	5.0	0.023	0.871	5726.3	8.0	44326.1	61.8	13537.7	146.2
		≥ 35 yr	5.0	0.021	0.879	6443.2	7.8	53455.1	64.6	14572.3	146.1
		≥ 30 yr	5.0	0.021	0.881	6737.6	7.7	56396.5	64.7	15225.1	146.1
	AC	Whole	5.0	0.126	0.631	515.4	24.2	1397.6	65.5	1170.9	148.8
		≥ 80 yr	5.0	0.260	0.557	378.1	45.8	853.5	103.4	559.6	153.0
		≥ 75 yr	5.0	0.260	0.640	449.7	45.2	1249.2	125.5	547.0	152.7
		≥ 70 yr	5.0	0.256	0.634	439.0	44.4	1200.5	121.3	551.5	152.4
		≥ 65 yr	5.0	0.240	0.634	433.8	42.0	1186.4	114.8	574.1	152.0
		≥ 60 yr	5.0	0.219	0.624	436.5	38.9	1161.3	103.4	639.4	151.5
		≥ 55 yr	5.0	0.197	0.621	445.2	35.4	1173.3	93.4	719.0	150.9
		≥ 50 yr	5.0	0.171	0.616	471.6	31.2	1229.4	81.5	868.1	149.9
		≥ 45 yr	5.0	0.146	0.626	493.5	27.4	1319.3	73.2	1006.5	149.3
		≥ 40 yr	5.0	0.134	0.628	508.3	25.5	1367.6	68.6	1103.8	149.0
		≥ 35 yr	5.0	0.129	0.629	513.2	24.6	1383.2	66.3	1151.7	148.9
		≥ 30 yr	5.0	0.126	0.631	516.1	24.2	1400.2	65.7	1168.9	148.8
CA19-9	Whole	Whole	37.0	0.032	0.789	1405.2	12.2	6656.1	57.7	3574.3	146.9
		≥ 80 yr	37.0	0.168	0.741	494.9	33.5	1908.7	129.3	584.4	152.7

		≥ 75 yr	37.0	0.164	0.735	505.6	32.7	1906.7	123.2	623.2	151.8
		≥ 70 yr	37.0	0.137	0.761	546.5	28.5	2288.5	119.3	691.5	150.9
		≥ 65 yr	37.0	0.115	0.762	589.9	25.0	2474.1	104.7	844.6	149.9
		≥ 60 yr	37.0	0.090	0.754	633.2	21.2	2568.7	85.8	1099.1	149.0
		≥ 55 yr	37.0	0.070	0.764	736.3	18.1	3114.0	76.7	1424.3	148.3
		≥ 50 yr	37.0	0.054	0.774	913.9	15.5	4035.0	68.4	1972.8	147.6
		≥ 45 yr	37.0	0.042	0.782	1108.4	13.6	5075.1	62.4	2614.6	147.2
		≥ 40 yr	37.0	0.036	0.785	1254.6	12.7	5833.6	59.1	3121.4	147.0
		≥ 35 yr	37.0	0.033	0.789	1359.0	12.3	6434.6	58.2	3427.4	146.9
		≥ 30 yr	37.0	0.032	0.789	1398.6	12.2	6634.7	57.8	3552.0	146.9
	HC	Whole	37.0	0.014	0.917	11789.9	9.3	142719.5	112.8	15277.9	146.1
		≥ 80 yr	37.0	0.143	0.500	622.7	29.7	1245.5	59.3	1565.3	149.1
		≥ 75 yr	37.0	0.078	0.833	2187.1	18.9	13122.9	113.1	2843.9	147.1
		≥ 70 yr	37.0	0.048	0.808	1207.9	14.5	6281.3	75.3	2356.2	146.9
		≥ 65 yr	37.0	0.043	0.783	1621.1	13.8	7457.1	63.3	3755.2	146.6
		≥ 60 yr	37.0	0.032	0.808	2252.2	12.1	11711.6	62.8	5254.5	146.4
		≥ 55 yr	37.0	0.026	0.853	3855.6	11.1	26218.2	75.5	7474.5	146.3
		≥ 50 yr	37.0	0.020	0.873	5259.2	10.2	41488.9	80.5	9558.2	146.2
		≥ 45 yr	37.0	0.016	0.899	7832.3	9.6	77452.6	95.3	12010.0	146.2
		≥ 40 yr	37.0	0.014	0.909	9540.9	9.4	104950.4	103.0	13537.7	146.2
		≥ 35 yr	37.0	0.014	0.915	10937.8	9.3	128950.9	109.7	14572.3	146.1
		≥ 30 yr	37.0	0.014	0.916	11530.0	9.3	137146.2	110.7	15225.1	146.1
	AC	Whole	37.0	0.082	0.763	663.4	20.0	2793.3	84.3	1170.9	148.8
		≥ 80 yr	37.0	0.170	0.747	488.5	33.8	1929.4	133.5	559.6	153.0
		≥ 75 yr	37.0	0.180	0.731	469.9	35.2	1749.5	131.1	547.0	152.7
		≥ 70 yr	37.0	0.171	0.757	502.4	33.7	2069.9	138.9	551.5	152.4
		≥ 65 yr	37.0	0.159	0.760	503.3	32.0	2093.5	133.2	574.1	152.0
		≥ 60 yr	37.0	0.144	0.748	496.7	29.7	1967.4	117.7	639.4	151.5
		≥ 55 yr	37.0	0.126	0.753	519.6	26.9	2105.0	109.0	719.0	150.9
		≥ 50 yr	37.0	0.109	0.759	581.1	24.2	2411.0	100.4	868.1	149.9
		≥ 45 yr	37.0	0.095	0.762	620.3	21.9	2601.4	92.0	1006.5	149.3
		≥ 40 yr	37.0	0.087	0.761	644.3	20.8	2694.8	87.0	1103.8	149.0
		≥ 35 yr	37.0	0.083	0.763	659.0	20.2	2780.6	85.2	1151.7	148.9
		≥ 30 yr	37.0	0.082	0.763	663.9	20.0	2805.1	84.5	1168.9	148.8
CA72-4	Whole	Whole	6.0	0.126	0.756	2584.5	25.9	10605.1	106.2	3574.3	146.9
		≥ 80 yr	6.0	0.194	0.790	676.1	37.1	3221.6	176.7	584.4	152.7
		≥ 75 yr	6.0	0.190	0.762	627.4	36.3	2641.0	152.8	623.2	151.8
		≥ 70 yr	6.0	0.180	0.755	651.5	34.8	2661.4	142.2	691.5	150.9
		≥ 65 yr	6.0	0.173	0.749	749.3	33.4	2980.7	133.0	844.6	149.9
		≥ 60 yr	6.0	0.166	0.745	933.2	32.3	3653.5	126.5	1099.1	149.0
		≥ 55 yr	6.0	0.158	0.746	1167.6	30.9	4599.8	121.6	1424.3	148.3
		≥ 50 yr	6.0	0.147	0.749	1552.7	29.1	6194.2	116.2	1972.8	147.6
		≥ 45 yr	6.0	0.139	0.753	2003.3	27.9	8106.4	112.8	2614.6	147.2

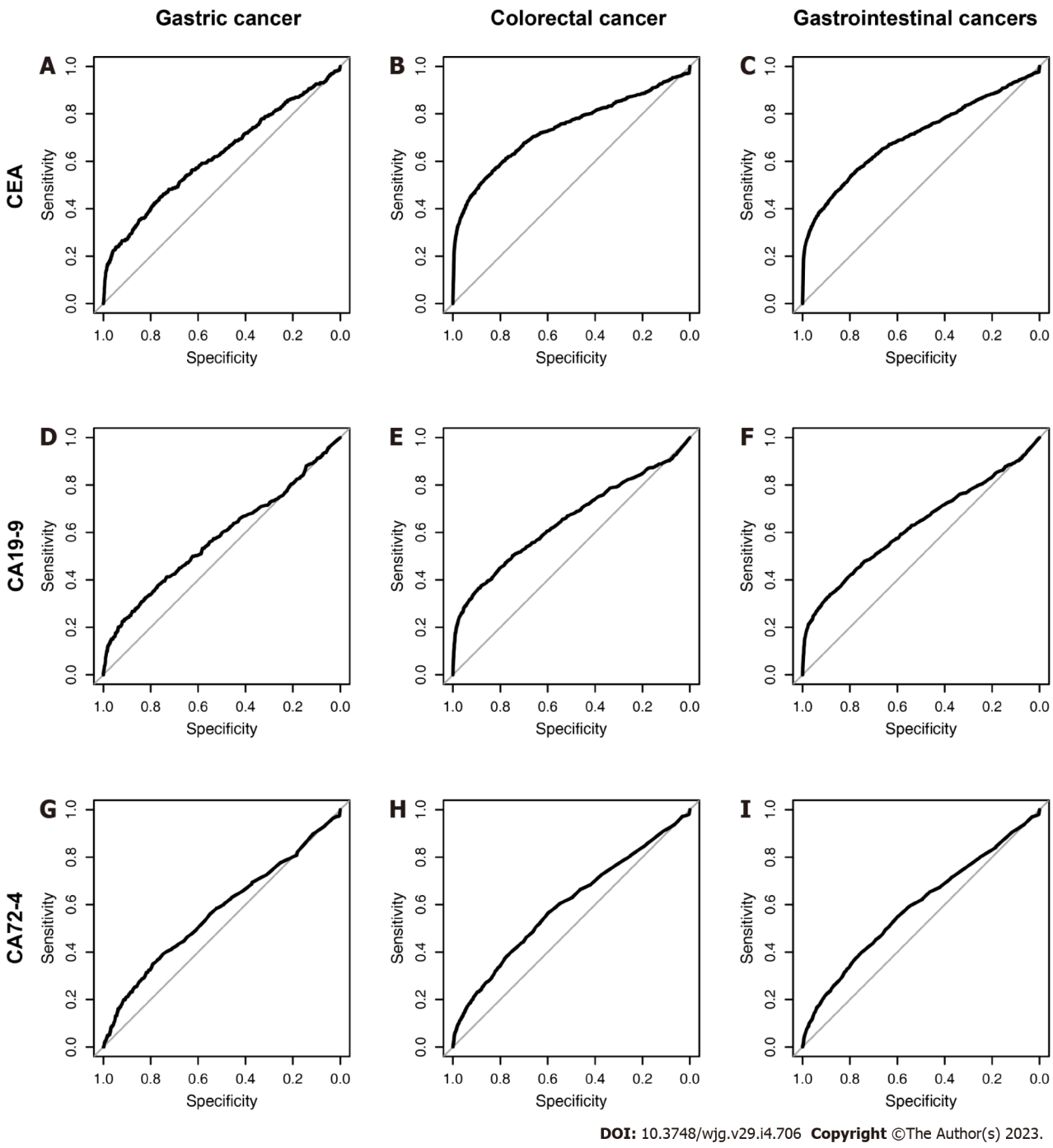
	≥ 40 yr	6.0	0.132	0.752	2293.2	26.7	9259.2	108.0	3121.4	147.0
	≥ 35 yr	6.0	0.128	0.755	2488.4	26.2	10145.7	106.7	3427.4	146.9
	≥ 30 yr	6.0	0.127	0.755	2565.2	25.9	10489.2	106.1	3552.0	146.9
HC	Whole	6.0	0.108	0.870	18401.4	23.0	141077.7	176.0	15277.9	146.1
	≥ 80 yr	6.0	0.333	0.500	1206.6	57.5	2413.3	114.9	1565.3	149.1
	≥ 75 yr	6.0	0.190	0.667	2042.4	35.2	6127.2	105.6	2843.9	147.1
	≥ 70 yr	6.0	0.144	0.808	2375.7	28.5	12353.8	148.1	2356.2	146.9
	≥ 65 yr	6.0	0.126	0.848	4345.5	25.8	28556.3	169.7	3755.2	146.6
	≥ 60 yr	6.0	0.118	0.821	4898.1	24.5	27289.4	136.5	5254.5	146.4
	≥ 55 yr	6.0	0.119	0.843	8033.4	24.7	51212.7	157.2	7474.5	146.3
	≥ 50 yr	6.0	0.118	0.866	11985.7	24.5	89577.1	183.4	9558.2	146.2
	≥ 45 yr	6.0	0.116	0.854	13653.0	24.3	93470.7	166.2	12010.0	146.2
	≥ 40 yr	6.0	0.111	0.861	15690.0	23.5	113076.5	169.4	13537.7	146.2
	≥ 35 yr	6.0	0.109	0.866	17224.4	23.1	128608.7	172.7	14572.3	146.1
	≥ 30 yr	6.0	0.108	0.867	18051.9	23.0	135991.3	173.3	15225.1	146.1
AC	Whole	6.0	0.177	0.733	997.5	33.8	3736.5	126.8	1170.9	148.8
	≥ 80 yr	6.0	0.183	0.797	643.0	35.6	3174.8	175.8	559.6	153.0
	≥ 75 yr	6.0	0.190	0.766	558.4	36.5	2383.4	155.8	547.0	152.7
	≥ 70 yr	6.0	0.194	0.751	539.5	37.2	2165.0	149.1	551.5	152.4
	≥ 65 yr	6.0	0.202	0.739	554.2	38.2	2126.5	146.7	574.1	152.0
	≥ 60 yr	6.0	0.212	0.736	634.8	39.7	2405.9	150.4	639.4	151.5
	≥ 55 yr	6.0	0.206	0.735	694.1	38.6	2617.8	145.6	719.0	150.9
	≥ 50 yr	6.0	0.194	0.732	793.2	36.7	2963.2	137.0	868.1	149.9
	≥ 45 yr	6.0	0.186	0.736	901.9	35.4	3410.7	133.8	1006.5	149.3
	≥ 40 yr	6.0	0.182	0.731	953.6	34.6	3547.9	128.7	1103.8	149.0
	≥ 35 yr	6.0	0.179	0.732	984.8	34.1	3674.4	127.3	1151.7	148.9
	≥ 30 yr	6.0	0.177	0.733	995.6	33.9	3723.6	126.8	1168.9	148.8

The economic indicators were total cost, cost per capita and their cost-effectiveness ratios. The units of carcinoembryonic antigen, carbohydrate antigen 19-9, and carbohydrate antigen 72-4 are μg/L, kU/L, and kU/L, respectively. C/E: Cost-effectiveness ratio; CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; CA72-4: Carbohydrate antigen 72-4; HC: Health checkup; AC: Active consultation.

In the active consultation subgroup, the total cost-effectiveness ratio in Scheme 2 was higher than that in Scheme 1 for all ages. CA19-9 and CA72-4 had higher total cost-effectiveness ratios in almost all subgroups, different from CEA (Figures 9 and 10). This also indicates that blood tests for the active consultation group are not enough and that the necessary gastrointestinal endoscopy procedure is more important.

DISCUSSION

This study included more than 32000 subjects who received CEA, CA19-9, CA72-4, gastroscopy and colonoscopy assessments. In our study, CEA and CA19-9 again have been proved to be ideal serum biomarkers for screening GICs. The specificity of CEA and CA19-9 was approximately 95.0%-97.5% at the traditional cut-off value, which was highly consistent with previous studies[5,6]. While for the diagnostic value of CA72-4, there is a discrepancy between the results of previous literatures and our clinical practice. In our study, the specificity of CA72-4 was less than 90%, indicating that the cut-off value could be higher, which made the sensitivity even lower. If the cut-off value of CA72-4 was 10, the sensitivity and specificity of GC were 0.163 and 0.933, respectively, and the sensitivity and specificity of CRC were 0.177 and 0.935, respectively.

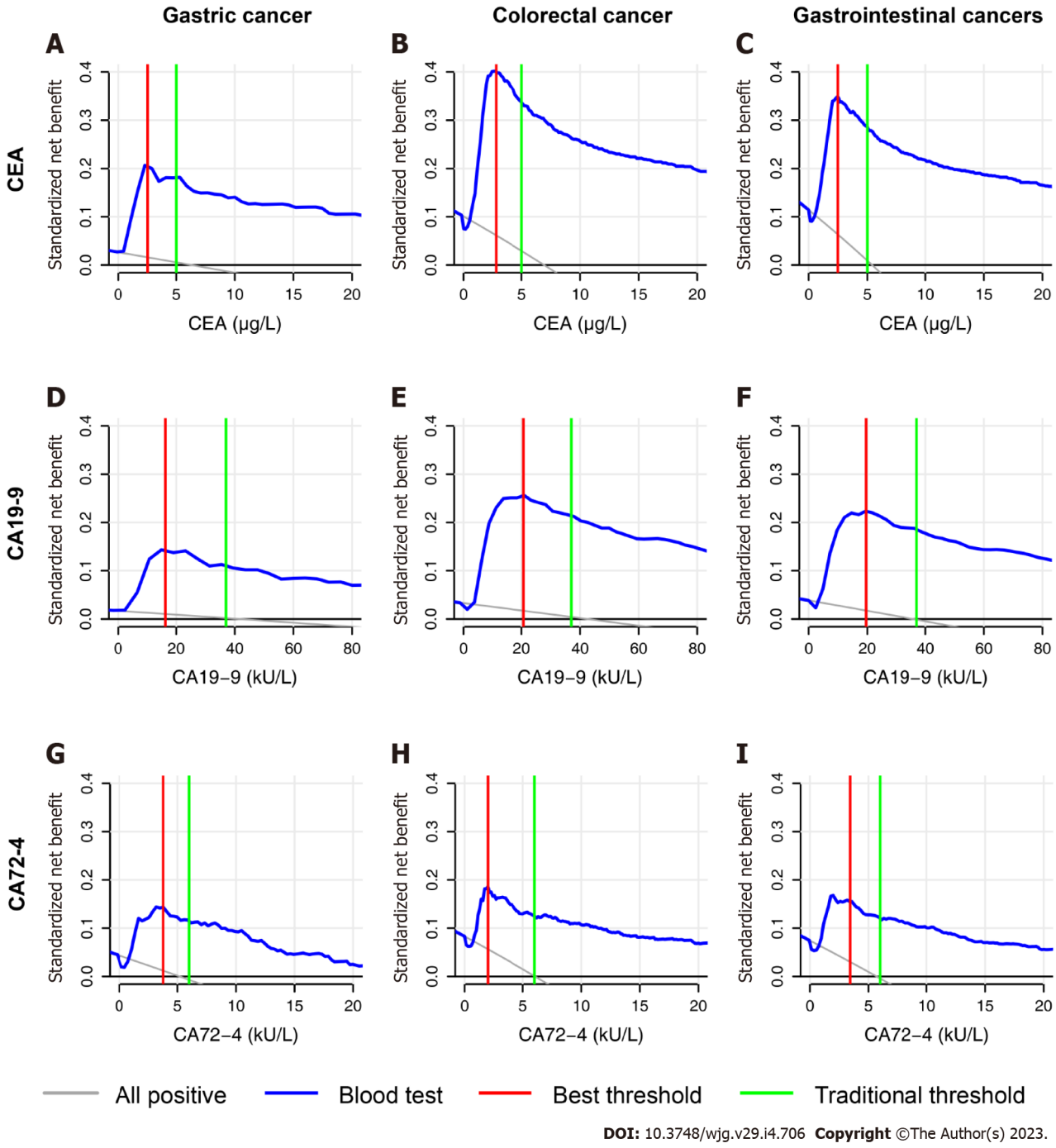


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Figure 5 Receiver operating characteristic curves of carcinoembryonic antigen, carbohydrate antigen 19-9, and carbohydrate antigen 72-4 for gastric cancer, colorectal cancer and gastrointestinal cancers. A-C: Carcinoembryonic antigen; D-F: Carbohydrate antigen 19-9; G-I: Carbohydrate antigen 72-4. CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9; CA 72-4: Carbohydrate antigen 72-4.

Besides the sensitivity and specificity, another important indicator is the PPV. Even for the best performing CEA, the PPV for GC was as low as 5.6% and that for CRC was only 21.2%. At the traditional cut-off value, the PPV of CA72-4 for GC was 2.3%, which meant that 97.7% of CA72-4-positive patients were false positive. The PPV also explained why there was no evidence of malignant disease in a large number of CA72-4-positive patients after a full set of auxiliary examinations. Of course, in view of the fact that the PPV is greatly affected by the prevalence, the real-world PPV would be lower. Therefore, our data on the predictive value is mainly used for comparison among the three biomarkers.

Several novel indicators are proposed to evaluate the economic value of blood markers for GICs. To calculate the economic value of a blood biomarker, it is inadequate to focus on the biomarker itself. A blood test is used as a screening test, and its significance also lies in the following gold standard test. By combining blood tests and endoscopy, the total cost and cost per capita of correctly diagnosing one case of GIC are excellent indicators, which are related to the cost, prevalence rate and sensitivity of blood

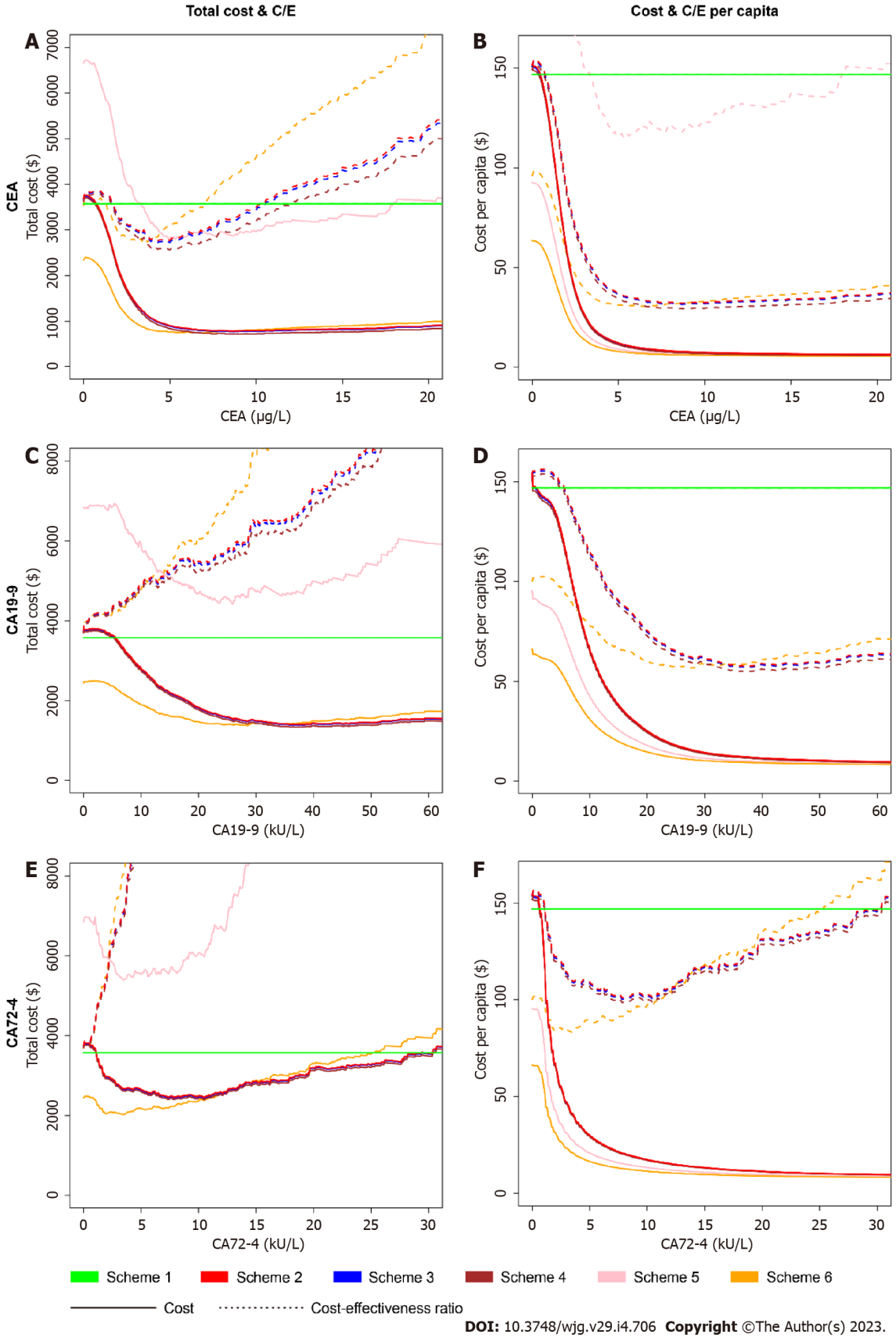


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Figure 6 Decision curves of tumor biomarkers for gastrointestinal cancers. A-C: Carcinoembryonic antigen; D-F: Carbohydrate antigen 19-9; G-I: Carbohydrate antigen 72-4. CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9; CA 72-4: Carbohydrate antigen 72-4.

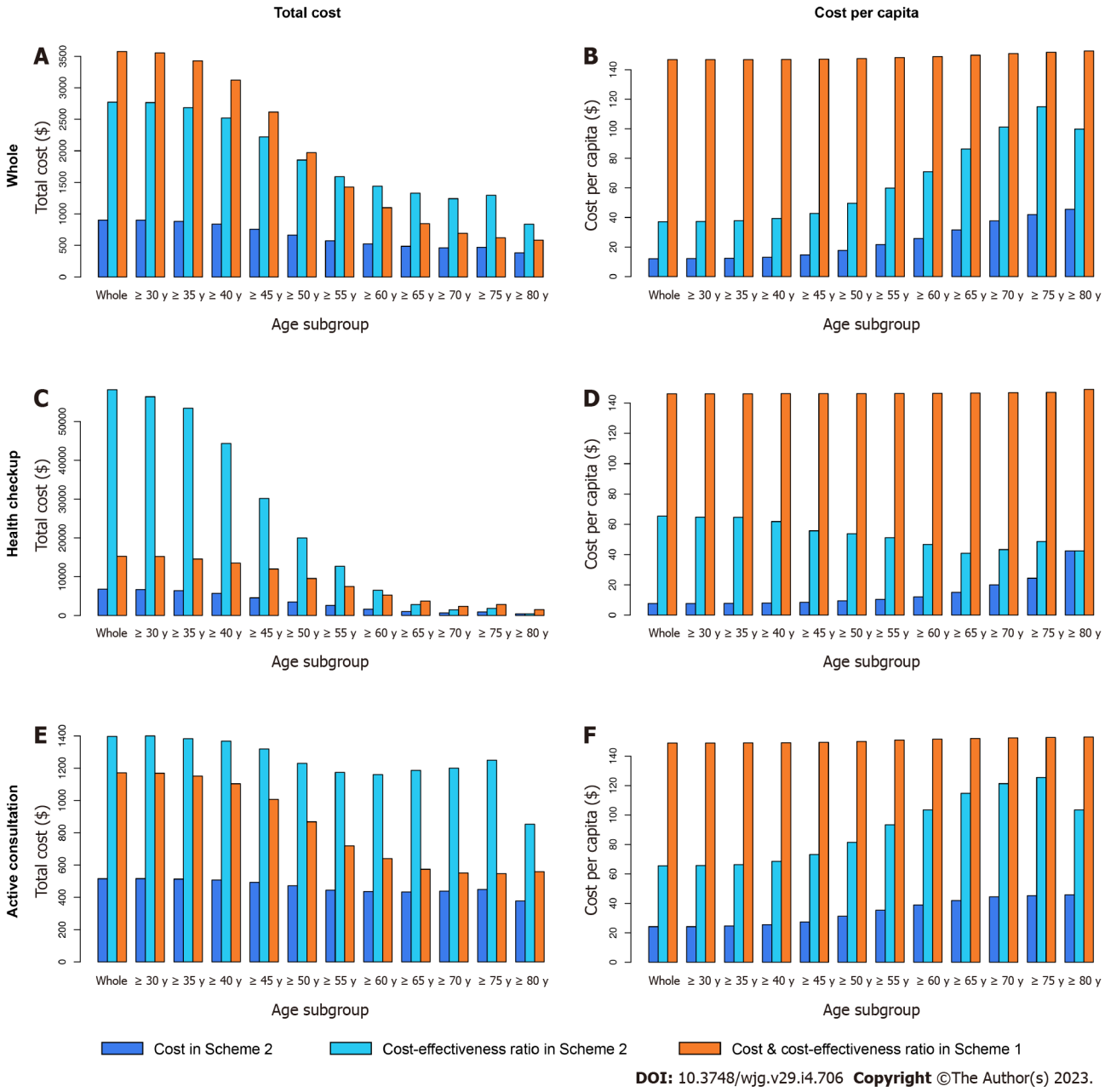
tests. However, these two indicators are not sufficient. If the prevalence of a disease increases, the cost per capita would also increase owing to more endoscopy examinations. It seemed that the cost increased, but the effect had actually improved even more. Therefore, it was necessary to calculate the cost-effectiveness ratio. What is the 'effect'? As a screening test, the sensitivity is its effect. The cost-effectiveness ratio is cost divided by sensitivity, which means the total cost for correctly diagnosing all subjects, including missed patients. We found that the total cost and the cost-effectiveness ratio per capita are positively correlated and change synchronously. Through our economic research, we have discovered the impacts of the order of gastrointestinal endoscopy and diagnostic thresholds on economic benefits. It is also clear that the economic value of combined blood biomarkers is not as good as that of the single CEA. Subgroup analysis shows that CEA had qualified diagnostic value for health checkup subjects above 65 years old.

In this study, only the subjects who received CEA, CA19-9, CA72-4, gastroscopy and colonoscopy were included. These inclusion criteria avoided or reduced several biases, such as workup bias, spectrum bias and measurement bias. For example, all of the included cases were examined by the gold



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Figure 7 Economic analysis of tumor biomarkers in six schemes for gastrointestinal cancers. A and B: Carcinoembryonic antigen; C and D: Carbohydrate antigen 19-9; E and F: Carbohydrate antigen 72-4. CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9; CA 72-4: Carbohydrate antigen 72-4.

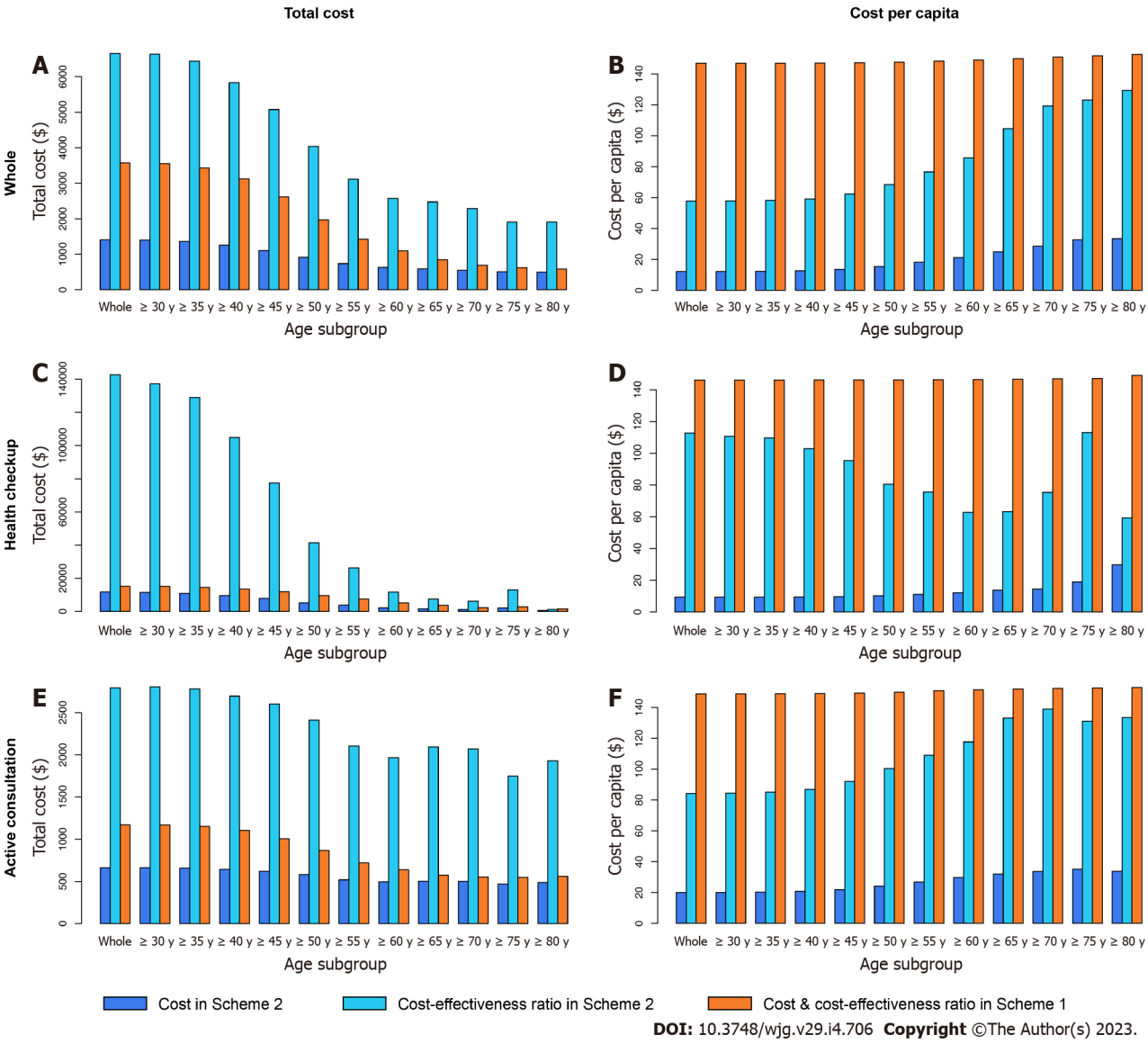


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Figure 8 Bar plots of subgroup analysis of economic indicators for carcinoembryonic antigen. A and B: Whole; C and D: Health checkup; E and F: Active consultation.

standard test, so there was no situation in which the subject with negative blood test results was not examined with the gold standard test. But on the other hand, the inclusion criteria led to an inevitable selection bias because the subjects undergoing gastrointestinal endoscopy are those with a high risk of digestive diseases, and the incidences of GC and CRC in this study were higher than that in the real world[15]. Many people undergo only blood tests but not gastrointestinal endoscopy when receiving a health checkup. As a result, some early GIC patients with normal CEA, CA19-9, CA72-4 Levels were not included. If these patients were included, the number of false negative subjects might have increased, and the sensitivity would have further decreased.

The advantages of this study are its continuous inclusion of subjects, use of the cohort study inclusion method (not case-control study), large sample size, inclusion of multiple tumors and use of multiple indicators. Especially for CA72-4 test, our sample size exceeded the sum of all previous reported studies. The comparison among multiple indicators highlighted the shortcomings of the diagnostic and economic value of CA72-4. In particular, the results of the classic markers CEA and CA19-9 were consistent with previous studies. We also proposed a new evaluation method for the economic efficiencies of tumor biomarkers for GIC and provided a reference for medical insurance policies.

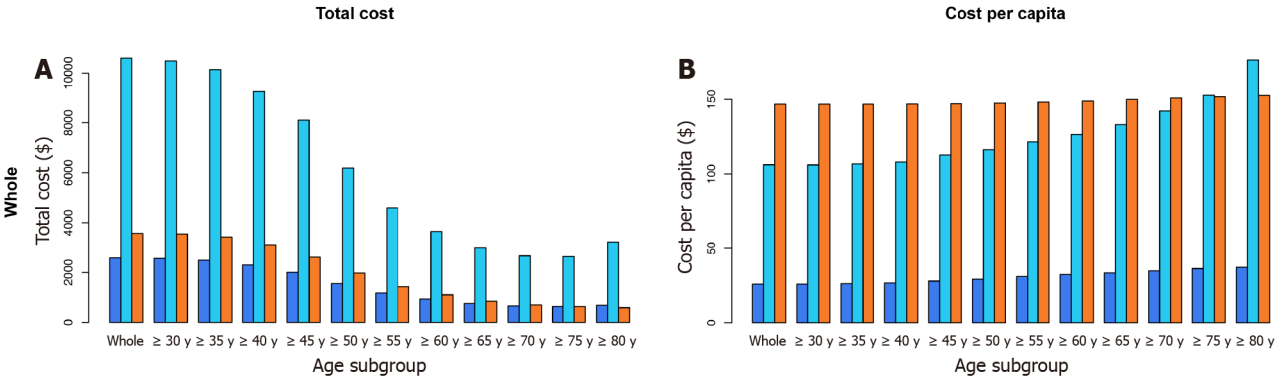


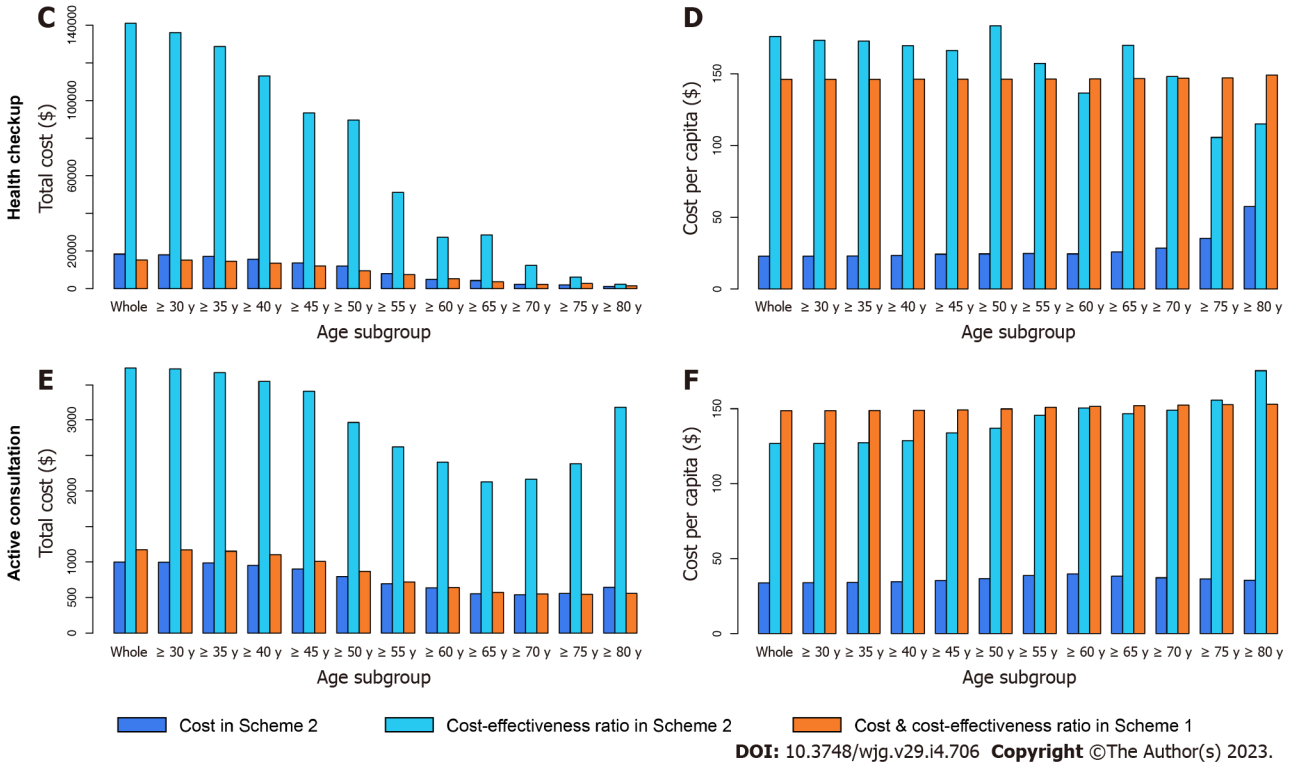
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Figure 9 Bar plots of subgroup analysis of economic indicators for carbohydrate antigen 19-9. A and B: Whole; C and D: Health checkup; E and F: Active consultation.

CONCLUSION

CEA had qualified diagnostic value for CRC and superior economic value for GICs, especially for health checkup subjects above 65 years old. CA72-4 was not suitable as a diagnostic biomarker.





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Figure 10 Bar plots of subgroup analysis of economic indicators for carbohydrate antigen 72-4. A and B: Whole; C and D: Health checkup; E and F: Active consultation.

ARTICLE HIGHLIGHTS

Research background

Studies showed that blood carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9) could be used to diagnose gastric cancer (GC) and colorectal cancer (CRC). Blood CA72-4 could be a potential biomarker to diagnose GC and CRC. A positive result in blood test would lead the subject to undergo further examinations.

Research motivation

Large-scale clinical application showed an extremely high false positive rate of CA72-4 for diagnosis, which leads to the waste of medical resources and heave social medical burden. The massive data and real-world diagnostic cohorts make it possible to further explore the diagnostic and economic value of biomarkers.

Research objectives

Through a real-world diagnostic cohort, we aimed to reassess the diagnostic and economic value of CEA, CA19-9, and CA72-4 for gastrointestinal malignant tumors in a large sample.

Research methods

Data from patients the medical examination center, outpatient department or inpatient department of Zhongshan Hospital of Fudan University from October 2006 to May 2018 were retrospectively evaluated. Four economic indicators were used to evaluate the economic value of tumor biomarkers. The diagnostic value of the three biomarkers was further evaluated.

Research results

The clinical benefits of CEA were higher than those of CA19-9, while the clinical benefits of CA72-4 were the lowest. The combination of biomarkers in the CRC and gastrointestinal malignant tumors significantly increased the AUC by less than 0.3, while that in GC did not. Compared to the economic indicators of the single biomarker CEA, the combination of biomarkers is not superior. At the threshold of 1.8 μg/L to 10.4 μg/L, all four indicators of CEA were lower than those in the scheme that conducted gastrointestinal endoscopy only. Subgroup analysis implied that the health checkup of CEA for people above 65 years old was economically valuable.

Research conclusions

CEA had qualified diagnostic value for CRC and superior economic value for gastrointestinal cancers, especially for health checkup subjects above 65 years old while CA72-4 was not suitable as a diagnostic biomarker.

Research perspectives

In real world, many people undergo only blood tests but not gastrointestinal endoscopy when receiving a health checkup. Those undergone gastrointestinal endoscopy were at a higher risk of digestive diseases, which leads to an inevitable selection bias. Future researches may emphasize on the involvement of patients with normal CEA, CA19-9, CA72-4 Levels to decrease the number of false negative subjects.

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FOOTNOTES

Author contributions: Liu HN, Yao C, and Wang XF contributed equally to this work; Liu HN, Wu H, and Liu TT conceived and designed the experiments; Liu HN, Yao C, and Wang XF drafted the manuscript; Liu HN, Yao C, Wang XF, and Pan D extracted the data; Liu HN, Zhang NP, and Chen YJ performed the statistical analyses; Zhao GP and Shen XZ revised the article; all authors finished reading and approving the final manuscript of this study.

Institutional review board statement: The study was reviewed and approved by the Zhongshan Hospital of Fudan University Institutional Review Board (Approval No. B2018-234).

Informed consent statement: The informed consent was waived from the patients.

Conflict-of-interest statement: All the authors have no conflict of interest related to the manuscript.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at liu.taotao@zs-hospital.sh.cn. Participants gave informed consent for data sharing.

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Country/Territory of origin: China

ORCID number: Hai-Ning Liu 0000-0001-7548-6269; Xiao-Fan Wang 0000-0001-5304-1883; Xi-Zhong Shen 0000-0003-3748-0709; Tao-Tao Liu 0000-0002-4623-9012.

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ORIGINAL ARTICLE

Retrospective Study

Feasibility and efficacy of endoscopic purse-string suture-assisted closure for mucosal defects induced by endoscopic manipulations

Ming-Ming Li, Yi Zhang, Fang Sun, Man-Xiu Huai, Fei-Yu Zhang, Chun-Ying Qu, Feng Shen, Zheng-Hong Li, Lei-Ming Xu

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Ming-Ming Li, Yi Zhang, Fang Sun, Man-Xiu Huai, Fei-Yu Zhang, Chun-Ying Qu, Feng Shen, Zheng-Hong Li, Lei-Ming Xu, Department of Gastroenterology, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200092, China

Corresponding author: Lei-Ming Xu, MD, Chief Doctor, Professor, Department of Gastroenterology, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, No. 1665 Kongjiang Road, Shanghai 200092, China. xuleiming@xinhuamed.com.cn

Abstract

BACKGROUND

Large or transmural defects induced by gastrointestinal endoscopic manipulations are difficult to close, although complete closure is recommended for better recovery. Endoscopic purse-string assisted suturing (EPSS) has been used in clinical practice and has proven to be an effective and safe technique for the closure of large mucosal defects. However, details regarding the efficacy of endoscopic pre-purse-string suture (P-EPSS) are unknown, especially that it offers several advantages over conventional EPSS (C-EPSS).

AIM

To elucidate the outcomes of EPSS-assisted closure in different clinical situations, and evaluate the efficacy of P-EPSS.

METHODS

This retrospective observational study included a total of 180 patients who underwent closure assisted by P-EPSS (n = 63) or C-EPSS (n = 117) between July 2014 and June 2020. The P-EPSS and C-EPSS groups were compared and the intergroup differences in aspects such as the lesion size, location, and morphology, incidence of complete closure, intraoperative perforation, and delayed adverse events were evaluated. Data on the features and clinical course of cases with adverse events were collected for further analysis.

RESULTS

Patients with lesion size larger than 3 cm, lesions located at the fundus of stomach, or submucosal tumors originating from the deep mucosa were more likely to undergo P-EPSS-assisted closure. The P-EPSS group showed a significantly higher proportion of intraoperative perforation (56% *vs* 17%) and a much

shorter procedure time (9.06 ± 6.14 min *vs* 14.84 ± 7.25 min). Among adverse events, the incidence of delayed perforation (5% *vs* 4%; P = 0.82) and delayed bleeding (3% *vs* 4%; P = 0.96) did not differ significantly between the groups. Multivariate analysis revealed that lesions with incomplete closure [odds ratio (OR) = 21.33; 95% confidence interval (CI): 5.45-83.45; P < 0.01] or size greater than 3 cm (OR = 3.14; 95%CI: 1.08-9.18; P = 0.039) showed a statistical tendency to result in an increase in delayed adverse events.

CONCLUSION

The present study revealed that EPSS could achieve secure complete closure of mucosal defect. P-EPSS could shorten the procedure and yield complete closure of mucosal defects. Rather than closure-type selection, incomplete closure or lesion size larger than 3 cm were associated with worse outcomes.

Key Words: Endoscopic purse-string suture; Mucosal defect; Endoscopic full-thickness resection; Endoscopic submucosal dissection

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Core Tip: Endoscopic purse-string assisted suturing (EPSS) has proven to be an effective and safe technique for the closure of large mucosal defects. Endoscopic pre-purse-string suture (P-EPSS) is recently introduced and offers several advantages over conventional endoscopic purse-string suture (C-EPSS). We found that the novel method could offer several advantages over C-EPSS. This retrospective observational study included a total of 180 patients who underwent P-EPSS (n = 63) or C-EPSS (n = 117), and evaluate the feasibility and efficacy of P-EPSS-assisted closure in different clinical situations. In conclusion, EPSS could achieve secure complete closure of mucosal defect. P-EPSS could shorten the procedure and yield complete closure of mucosal defects rather than the C-EPSS closure-type.

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INTRODUCTION

The endoscopic purse-string assisted suturing (EPSS) method has been used in clinical practice for nearly 20 years and has proven to be an effective and safe technique for the closure of large mucosal defects induced by endoscopic manipulations[1-5]. Inspired by the surgical purse-string suture strategy, an endoloop and repositionable clips have been successfully combined to manage intraluminal wounds *via* an endoscope[6-10]. However, some defects are difficult to resect and are expected to induce perforation during endoscopic manipulations, necessitating the application of an endoscopic pre-purse-string suture (P-EPSS) procedure. This novel suture method was first described by Wu *et al*[11] and was usually applied in cases involving exposed endoscopic full-thickness resections (EFTRs). At our medical center, we expanded the indications for P-EPSS and attempted to use it in more procedures such as endoscopic submucosal dissection (ESD) and endoscopic submucosal excavation (ESE) of big defects, potentially simplifying the process of closure and avoiding the possibility of postoperative adverse events.

To our knowledge, no previous reports have described the advantages of P-EPSS over conventional EPSS (C-EPSS). Furthermore, previous clinical studies on the application of EPSS usually had small sample sizes and mainly focused on providing detailed descriptions of endoscopic procedures rather than assessing the feasibility and efficacy of the technique[12-15]. Therefore, we conducted a large-scale analysis of case series focusing on the effectiveness and safety of EPSS. We compared the differences between the P-EPSS and C-EPSS group in many aspects and tried to illustrate the considerations involved in choosing the appropriate closure type according to the defect's clinical characteristics.

MATERIALS AND METHODS

Study design

This was a retrospective observational study performed in accordance with the Helsinki Declaration. It was approved by the Ethics Committee at Xinhua Hospital (approval number: XHEC-C-2018-109), Shanghai Jiaotong University School of Medicine. Written informed consent for the procedures was obtained from all patients.

Eligible patients

A total of 180 consecutive patients with large mucosal defects who underwent C-EPSS or P-EPSS at our institute from July 2014 to June 2020 were included in the study. We compared the findings between the P-EPSS group (n = 63) and C-EPSS group (n = 117) for further analysis. All patients had undergone different endoscopic manipulations (ESD, ESE, or exposed EFTR), and the endoscopic suture method was applied to close large mucosal defects with or without transmural defects. All the procedures were completed by four chief physicians who had more than 10 years of experience in performing endoscopic operations. The patient database included data pertaining to patient demographics, location and size of the mucosal defects after resection, procedure time, successful closure rate, delayed adverse events, duration of hospitalization, and total cost of hospitalization.

C-EPSS and P-EPSS procedures

The endoscopic manipulation always started with the mucosa marking procedure, in which markings were placed 3 to 5 mm outside the circumference of the lesion. After injection of epinephrine diluted in saline solution (1:100000) into the submucosal layer to raise the submucosa, endoscopists performed the resection procedure. The C-EPSS procedure is a common method used for closure of defects. After dissection of precancerous lesions or tumor specimens, an endoloop and clips were inserted simultaneously into the location of defect through the therapeutic endoscope for complete closure. Single-channel or double-channel endoscopy was used according to the specific defect status. The endoloop was anchored onto the full thickness of the defect's distal margin with the clip, followed by insertion of three to six additional clips to anchor the endoloop at different sides of the margin. Finally, the endoloop was tightened by slightly pulling all the edges together. Other clips were used if any clip was not accurately positioned or the purse-string suture was not tight.

The P-EPSS procedure was always used when defects were difficult to resect and expected to induce perforation. In these cases, to reduce difficulties in manipulation in the suturing process, decrease the entry of gas into the abdominal cavity, and avoid postoperative adverse events, the endoloop and clips were advanced around the defect before the end of the dissection phase; this procedure was routinely performed with one or two clips. The remaining clips were anchored around the defects when the lesions were resected. Tightening of the endoloop was immediately performed after placement of the clips. This procedure was named P-EPSS. An example of the closure procedure is shown in Figures 1A-I and the Video(Supplementary material).

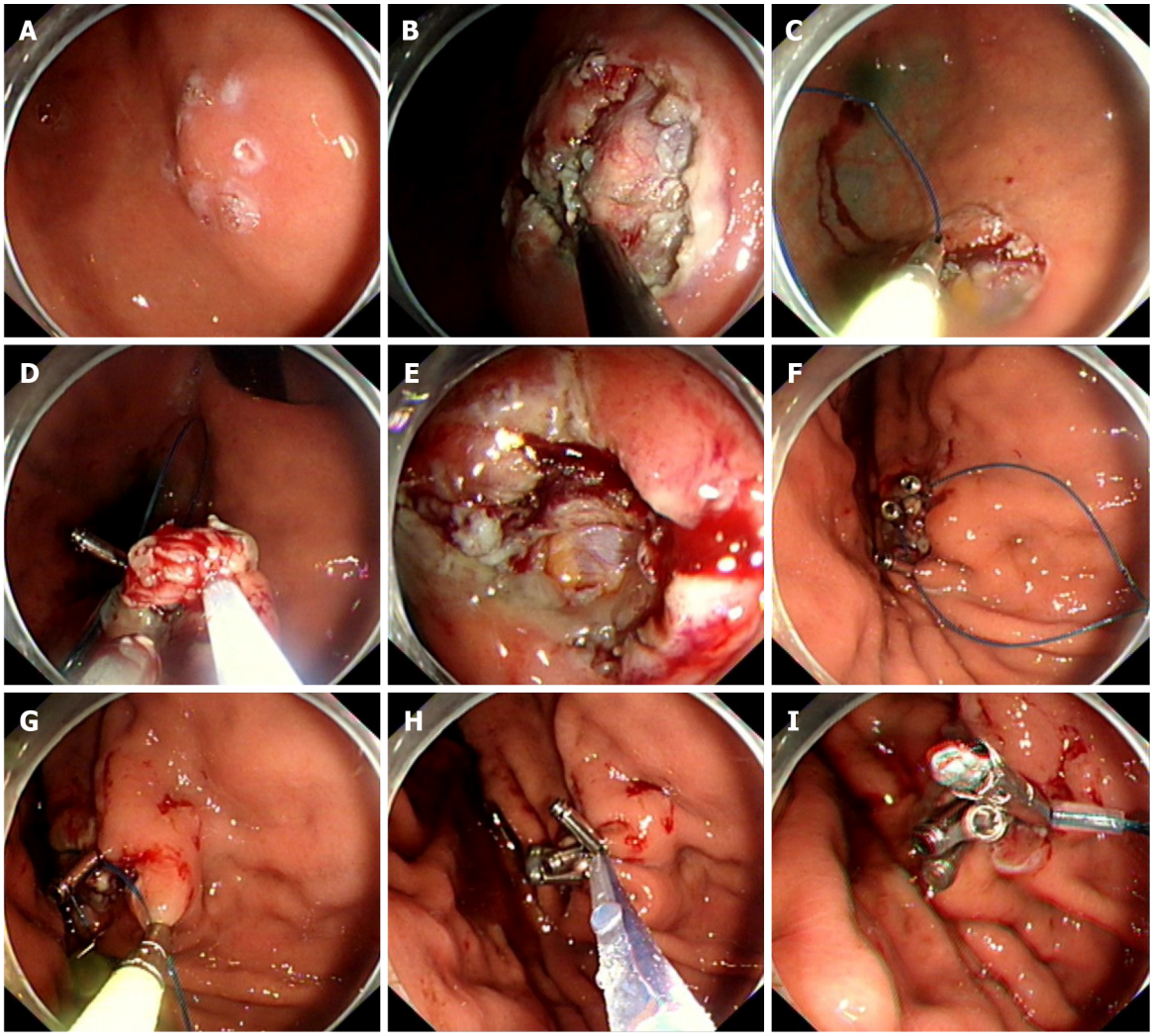
The procedure time of the P-EPSS-assisted closure was defined as the time from the usage of the first clip in the operation to completion of the closure, but the time required for the resection procedure was excluded (as shown in Figures 1C and 1F-I). Exclusion of the time required for the resection procedure was considered appropriate due to the differences in the difficulty levels in the operation.

Postoperative management

After the manipulations, the patients received drug therapy and were fasted for 2-4 d; body temperature was monitored daily, and postoperative adverse events were recorded as required. After evaluating the results of blood examinations and abdominal radiographs, the patients were allowed to drink, which was followed by a liquid diet. Proton pump inhibitors (rabeprazole 20 mg/d, lansoprazole 30 mg/d, or esomeprazole 20 mg/d) were administered for 1 mo if the patients had undergone upper gastrointestinal (GI) tract surgeries. Prophylactic antibiotics were routinely used in patients who showed intraoperative perforation and in patients with elevated white blood cell counts. If the patients showed good outcomes, they were discharged after 3-5 d. The first clinical follow-up was performed 3-6 mo after the manipulation, and then annually after the first year. The follow-up assessments included evaluation of digestive symptoms and gastro/enteroscopy.

Measured outcomes

The success rate of complete closure, incidence of adverse events (delayed perforation and bleeding), procedure time, duration of hospitalization, and total costs were analysed as outcomes of EPSS-assisted closure of mucosal defects and compared them between the P-EPSS and C-EPSS groups. The definition of perforation required elucidation in advance and included intraoperative and delayed perforation. The former was defined as a perforation observed before the end of the endoscopic manipulation, indicating the formation of a transmural defect during the operation; the latter was defined as any perforation found thereafter. Delayed bleeding was defined as hematemesis or melena requiring endoscopic hemostasis after the procedure. We did not treat adverse events occurring during the



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Figure 1 Schematic figure of the pre-endoscopic purse-string assisted suturing procedure. A: Mucosa marking step: Dots were marked around the tumor; B: Expose the lesion; C: Pre-endoscopic purse-string assisted suturing by using an endoloop and a metal clip; D and E: Intraoperative perforation in exposed endoscopic full-thickness resection; F-I: Immediate closure of the perforation by tightening endoloop.

endoscopic procedure, such as intraoperative perforation in exposed EFTR, as events; only delayed adverse events such as perforations or bleeding appearing after the end of closure were treated as events. We collected data on the features and clinical course of cases with adverse events, and analyzed associations between the unsatisfactory outcomes and defect features (complete closure or not, location, size, morphology, and closure type).

Statistical analyses

Categorical variables were analyzed using Pearson's chi-squared test or Fisher's exact test, as appropriate. For instance, as listed in Table 1, we used Pearson's chi-squared test to analyze the predictors when using the P-EPSS method. Continuous variables were analyzed using the student *t* test. The data for duration and cost of hospitalization were analyzed and summarized in Table 2. *P* values < 0.05 were considered statistically significant. All data were analyzed using the SPSS for Windows statistical software package (version 22.0; SPSS, Chicago, IL, United States).

RESULTS

Baseline characteristics

Patient details are described in Table 3. During the study period, 117 patients underwent C-EPSS, while 63 patients underwent P-EPSS for closure of defects induced by endoscopic manipulations. The ratio of different endoscopic manipulations (exposed EFTR:ESE:ESD) used in the study was approximately 1:1:3 (36:32:112), but this ratio showed no significant difference between the P-EPSS and C-EPSS groups (*P* >

Table 1 Predictors for closure of mucosal defects using endoscopic pre-purse-string suture method

Predictors	P-EPSS/C-EPSS (*n*)	Odds ratio	95%CI	*P* value
Location (%)				
Fundus	35/44	2.07	1.11-3.86	0.03[1]
Others	28/73	1		
Defect size				
≥ 3 cm	44/8	31.55	12.86-77.40	< 0.01[1]
< 3 cm	19/109	1		
Morphology				
Deep SMT	36/40	2.57	1.37-4.81	< 0.01[1]
Others	27/77	1		

[1]Statistically significant.
P-EPSS: Endoscopic pre-purse-string suture; C-EPSS: Conventional endoscopic purse-string suture; SMT: Submucosal tumor; CI: Confidence interval.

Table 2 Outcomes of purse-string suture according to different closure type

Outcomes	Total	P-EPSS	C-EPSS	*P* value
Complete closure, *n* (%)	169 (94)	59 (94)	110 (94)	0.80
Intraoperative perforation, *n* (%)	55 (31)	35 (56)	20 (17)	< 0.01[1]
Delayed adverse events, *n* (%)				
Delayed perforation	8 (4)	3 (5)	5 (4)	0.82
Delayed bleeding	7 (4)	2 (3)	5 (4)	0.96
Operation time (min), mean ± SD	12.82 ± 7.83	9.06 ± 6.14	14.84 ± 7.25	< 0.01[1]
Fasting period (d), median (range)	2 (1-5)	2 (1-5)	2 (1-5)	0.88
Hospital stay (d), median (range)	6 (4-11)	6 (4-10)	6 (4-11)	0.87
Total cost (dollars), mean ± SD	2481 ± 445	2448 ± 365	2498 ± 418	0.47

[1]Statistically significant.
SD: Standard deviation; P-EPSS: Endoscopic pre-purse-string suture; C-EPSS: Conventional endoscopic purse-string suture.

0.05). In terms of the location, more than three-quarters of the defects were located in the stomach, especially in the part of the fundus (44%). The mean defect size was 2.5 ± 1.46 cm. The difference in defect size between the P-EPSS and C-EPSS groups was significant (3.6 ± 1.13 cm *vs* 2.5 ± 0.68 cm, $P < 0.05$). In terms of defect morphology, more than 70% of the defects were submucosal tumors; 42% were submucosal tumors (SMTs) located in the deep muscularis propria and 31% were superficial SMTs. The size and morphology of the defects in the two groups were different, and a detailed analysis of these characteristics is provided in the next section.

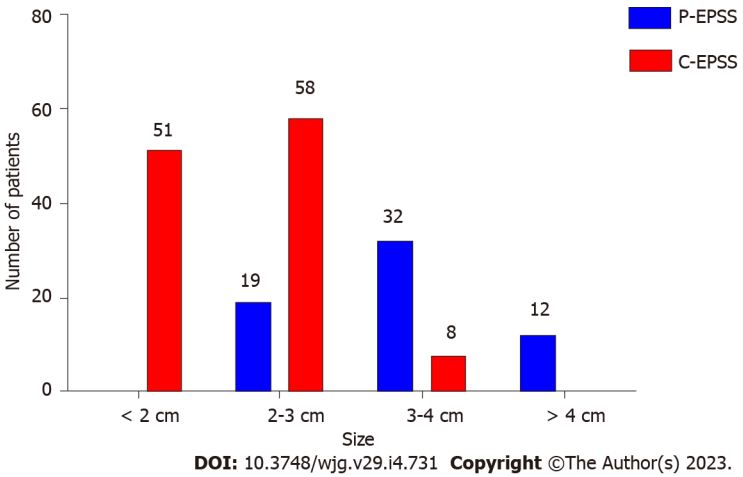
P-EPSS predictors related to the defect's clinical features

As mentioned above, many factors, including the defect's position, size, and morphology, could indicate and predict the choice of the P-EPSS method. In terms of position, more than half (54%) of the defects in the P-EPSS group were located at the fundus of the stomach, which was much higher than the corresponding proportion in the C-EPSS group (38%). In terms of the size, the proportion of cases with defects larger than 3 cm in the P-EPSS group was significantly more than the proportion of cases with defects < 3 cm in size ($P < 0.01$), indicating a significant trend toward the use of P-EPSS to close large defects. We designed a histogram to illustrate the choice of closure type according to the defect size, as shown in Figure 2. In terms of the morphology, no significant increase was observed in the proportion of cases involving P-EPSS in relation to the morphology of SMTs (33% *vs* 41%, $P > 0.05$). The difference between the P-EPSS and C-EPSS groups was significant only for patients with SMTs originating from the muscularis propria (57% *vs* 34%, $P < 0.01$). In terms of differences in endoscopic manipulations, no significant increase was observed in the proportion of P-EPSS cases in the exposed EFTR group (42% *vs*

Table 3 Baseline characteristics of the cases in this study

Patients detail	Total (*n* = 180)	P-EPSS (*n* = 63)	C-EPSS (*n* = 117)	*P* value
Age (yr), mean (range)	60 (38-78)	62 (42-76)	60 (38-78)	0.19
Sex, *n* (%)				0.75
Female	112 (62)	38 (60)	74 (63)	
Male	68 (38)	25 (40)	43 (37)	
Manipulation, *n* (%)				0.40
EFTR	36 (20)	15 (24)	21 (18)	
ESE	32 (18)	13 (21)	19 (16)	
ESD	112 (62)	35 (55)	77 (66)	
Location, *n*(%)				0.10
Stomach				
Fundus	79 (44)	35 (56)	44 (38)	
Body	45 (25)	11 (17)	34 (29)	
Antrum	22 (12)	8 (13)	14 (12)	
Colon and rectum	34 (19)	9 (14)	25 (21)	
Defect size (cm), mean ± SD	2.89 ± 1.46	3.6 ± 1.13	2.5 ± 0.68	0.02[1]
Morphology, *n*(%)				0.01[1]
Deep SMT	76 (42)	36 (57)	40 (34)	
Superficial SMT	55 (31)	15 (24)	40 (34)	
Precancerous lesions	49 (27)	12 (19)	37 (32)	

[1]Statistically significant.
SD: Standard deviation; SMT: Submucosal tumor; ESD: Endoscopic submucosal dissection; ESE: Endoscopic submucosal excavation; EFTR: Endoscopic full-thickness resection; P-EPSS: Endoscopic pre-purse-string suture; C-EPSS: Conventional endoscopic purse-string suture.



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Figure 2 Histogram of the choice of closure type according to the defect size. P-EPSS: Endoscopic pre-purse-string suture; C-EPSS: Conventional endoscopic purse-string suture.

33%, $P > 0.05$).

Next, we performed multivariate analysis to identify predictors for P-EPSS. Defect location at the fundus of the stomach [odds ratio (OR) = 2.07; 95% confidence interval (CI): 1.11-3.86; $P = 0.03$], defect size larger than 3 cm (OR = 31.55; 95%CI: 12.86-77.40; $P < 0.01$), and SMTs originating from the deep mucosa (OR = 2.57; 95%CI: 1.37-4.81; $P < 0.01$) were significantly associated with a high rate of P-EPSS (Table 1).

Comparison of treatment outcomes between the P-EPSS and C-EPSS groups

Table 2 presents a comparison of treatment outcomes between the P-EPSS and C-EPSS groups. The proportion of complete closure assisted by purse-string sutures was 94% and was similar between the two groups. Patients with incomplete closure received conservative treatment and eventually recovered from the trauma. None of them required alternative surgical repair procedures. While more patients from the P-EPSS group were inclined to experience intraoperative perforation (56% *vs* 17%, $P < 0.01$), the procedure time was significantly shorter in the P-EPSS group (9.06 ± 6.14 min *vs* 14.84 ± 7.25 min, $P < 0.01$).

In the assessment of adverse events, the two groups showed no significant differences in the proportions of delayed perforation (5% *vs* 4%; $P = 0.82$) and delayed bleeding (3% *vs* 4%; $P = 0.96$). Cases of adverse events were successfully managed by endoscopic treatment and conservative therapy. After the treatment, the fasting period, duration of hospitalization, and total cost of hospitalization were similar between the two groups.

Analysis of risk factors for delayed adverse events

The features and clinical course of the 15 cases showing delayed adverse events are shown in Table 4, and the results of the logistic regression analysis are shown in Table 5. Multivariate analysis revealed that incomplete closure of the defect was the main independent predictor for an increased number of delayed perforation or bleeding events, and it showed approximately 95% significant increase in delayed adverse events (OR = 21.33; 95%CI: 5.45-83.45; $P < 0.01$). Moreover, cases with defect size more than 3 cm showed a statistical tendency toward an increase in delayed adverse events (OR = 3.14; 95%CI: 1.08-9.18; $P = 0.039$). However, defect position (fundus or others) or morphology (SMT or precancerous lesions) as well as the closure type selected (P-EPSS or C-EPSS) were not related to significant differences in delayed adverse events.

DISCUSSION

With the development and popularization of endoscopic techniques, more GI diseases can be detected early and manipulated using minimally invasive methods[16-18]. GI endoscopy procedures such as ESD, ESE, EFTR, and submucosal tunnel endoscopic resection are widely used for the treatment of precancerous lesions or SMTs within the GI tract[19-23]. However, their increasing usage has led to the problem of achieving successful complete closure of the mucosal defects induced by different endoscopic manipulations.

Clips can be applied to effectively manage small defects, but they are too small to work well for large mucosal defects. More importantly, tissue approximation with full-thickness closure is technically impossible with clips, and clips may prematurely drop off the mucosa due to peristalsis and the radial forces of large post-EFTR defects, resulting in delayed perforation and severe complications. Furthermore, clip-closure methods appear to be strongly operator-dependent[9,24,25].

These factors have necessitated the development of new techniques. As reported in one study, various techniques have been used for closure of large defects (especially for exposed defects after EFTR), and these mainly consist of clip- and endoloop-assisted closure methods[26]. For instance, multiple studies reported that over-the-scope-clip (OTSC) application, which can close defects with serosa-to-serosa apposition, unlike mucosa-to-mucosa apposition, can successfully close defects with long-term reliability[27-30]. However, OTSCs can close GI defects only up to 2.5-3 cm in size[31,32], and the OTSC system shows limited effectiveness in some anatomic sites, such as the pylorus and the proximal esophagus. In addition, the edema and tissue folding associated with the usage of OTSCs can potentially narrow the lumen[33], and the OTSC technique is expensive, which can increase the financial burden on patients.

Purse-string assisted suturing has been widely used and is a financially feasible approach in cases with large defects induced by endoscopic manipulations. However, previous studies on the efficacy of EPSS methods evaluated a limited source of cases. Moreover, most of these studies focused on providing a detailed introduction of the procedure for purse-string sutures rather than performing a detailed analysis of its feasibility and efficacy. Wang *et al*[12] reported that the purse-string suture for colonic mucosal defects could be successfully completed using a single-channel endoscope. They described the detailed procedure of the EPSS method and concluded that no severe complications were recorded in all 18 cases. Kato *et al*[34] summarized their findings for duodenal ESD and found that complete closure of the mucosal defect by the EPSS method was relatively easy and involved a reduced delayed adverse event rate. Other related studies always introduced the use of this technique for some specific conditions, such as iatrogenic perforations in the colon or duodenum. Ryu *et al*[13] performed the purse-string suture technique to close iatrogenic colon perforations in eight cases and verified that EPSS can be appropriate for closing large colon perforations. In their analyses of the data obtained from 23 cases involving the closure of large iatrogenic duodenal perforations with purse-string sutures, Zhu *et al*[14] concluded that the EPSS method was feasible, effective, and easy for closure of perforations. Furthermore, some other studies focused on endoloop-assisted closure of exposed defects after EFTR

Table 4 Clinical features of 15 patients with adverse events

Age (yr)	Sex	Manipulation	Location	Size (cm)	Morphology	Closure type	Complete closure	Intraoperative perforation	Procedure time (min)	Delayed adverse events	Discharge (d)	Total cost (dollars)
60	Male	ESD	Fundus	3	Superficial SMT	C-EPSS	Yes	Yes	20	Delayed bleeding	10	2522
58	Male	EFTR	Fundus	4	Deep SMT	P-EPSS	Yes	Yes	15	Delayed perforation	11	2835
75	Female	ESD	Rectum	2.5	Superficial SMT	C-EPSS	No	No	22	Delayed bleeding	9	2710
56	Female	ESE	Fundus	3.5	Deep SMT	P-EPSS	No	No	24	Delayed perforation	6	2238
74	Female	ESD	Antrum	2	Precancerous lesion	C-EPSS	Yes	No	20	Delayed bleeding	7	2489
68	Male	ESD	Body	2.5	Deep SMT	C-EPSS	No	No	10	Delayed perforation	8	2470
58	Male	EFTR	Fundus	4	Superficial SMT	P-EPSS	Yes	Yes	15	Delayed perforation	7	2556
62	Male	ESE	Rectum	3.5	Precancerous lesion	C-EPSS	No	No	20	Delayed perforation	10	2884
63	Female	ESD	Antrum	3.5	Precancerous lesion	C-EPSS	No	No	12	Delayed bleeding	8	2690
71	Male	ESD	Rectum	2.5	Precancerous lesion	C-EPSS	Yes	No	14	Delayed perforation	6	2729
69	Male	ESE	Colon	2	Precancerous lesion	C-EPSS	Yes	No	18	Delayed perforation	8	2577
76	Male	EFTR	Fundus	3.5	Deep SMT	P-EPSS	Yes	Yes	25	Delayed perforation	8	2306
56	Female	ESD	Fundus	2.5	Superficial SMT	C-EPSS	Yes	Yes	10	Delayed bleeding	7	2423
61	Male	EFTR	Fundus	2.5	Deep SMT	C-EPSS	No	Yes	15	Delayed perforation	6	2789
70	Female	EFTR	Body	3.5	Superficial SMT	P-EPSS	Yes	Yes	20	Delayed bleeding	7	2687

SMT: Submucosal tumor; P-EPSS: Endoscopic pre-purse-string suture; C-EPSS: Conventional endoscopic purse-string suture; ESD: Endoscopic submucosal dissection; ESE: Endoscopic submucosal excavation; EFTR: Endoscopic full-thickness resection.

[35-39]. In comparison with other closure methods, the adoption in combination with endoclips may allow the management of larger post-EFTR defects and may reinforce the wound closure[24,40].

To the best of our knowledge, the present study is the largest case series exploring the effectiveness and safety of the EPSS method. A total of 180 cases involving EPSS-assisted closure performed at our medical center were enrolled in this study for further analysis. More importantly, our study was the first to compare the feasibility and efficacy of different closure types (P-EPSS and C-EPSS) used in endoscopic manipulations.

Table 5 Risk factors for delayed adverse events

Factors	Total (AE)	Odds ratio	95%CI	*P* value
Closure of defects				
Incomplete	11 (6)	21.33	5.45-83.45	< 0.01[1]
Complete	169 (9)	1		
Location (%)				
Fundus	79 (7)	1.13	0.39-3.26	0.82
Others	101 (8)	1		
Defect size				
≥ 3 cm	52 (8)	3.14	1.08-9.18	0.039[1]
< 3 cm	128 (7)	1		
Morphology				
Deep SMT	76 (6)	0.90	0.31-2.66	0.86
Others (superficial SMT and precancerous lesion)	104 (9)	1		
Closure type				
P-EPSS	63 (5)	0.92	0.30-2.83	1.00
C-EPSS	117 (10)	1		
Intraoperative perforation				
Yes	55 (7)	2.13	0.73-6.21	0.24
No	125 (8)	1		
Dissection method				
EFTR	36 (5)	2.16	0.69-6.78	0.19
Others (ESD and ESE)	144 (10)	1		

[1]Statistically significant.

SMT: Submucosal tumor; P-EPSS: Endoscopic pre-purse-string suture; C-EPSS: Conventional endoscopic purse-string suture; AE: Adverse event; ESD: Endoscopic submucosal dissection; ESE: Endoscopic submucosal excavation; EFTR: Endoscopic full-thickness resection.

P-EPSS has been employed as a novel closure method at our institution, accounting for 63 cases between 2014 and 2020. The previous study mainly used P-EPSS in the treatment of gastric tumors originating from the muscularis propria or gastric extra-luminal growth tumors[11]. In this study, we expanded the indications of P-EPSS and used this novel closure method in more situations, such as ESD or ESE cases with big defects. This approach is especially appropriate for defects characterized by a large size and specific positions or morphologies (*e.g.*, fundus or deep SMTs). All of the above clinical characteristics point to the same tendency, *i.e.*, intraoperative perforation. Thus, it is difficult and time-consuming to achieve complete closure, highlighting the need for careful selection of cases treated with P-EPSS-assisted closure.

In the present study, the P-EPSS group showed a significantly higher incidence of intraoperative perforation and a significantly shorter procedure time in comparison with the C-EPSS group. In this regard, the approach used to measure the procedure time of the EPSS method requires elucidation in advance. The time required for the resection procedure should be excluded for fairness, because the difficulty of endoscopic resection is different. Since the endoscopist had to take more time and effort to achieve complete closure of transmural defects, most of them would prefer to avoid the possibility of perforation in manipulations. Unexpectedly, the novel P-EPSS method could solve this problem from a different perspective by turning passive perforation to active perforation, and thereby providing sufficient time for preparation of perforation closure. Subtle and sensitive handling of the endoscope is essential for accomplishing the manipulation in EPSS. Although closure of the defects of transmural GI lesions revealed clinical effectiveness, this approach was not technically easy. The maneuverability of the endoscope was poor, especially in portions with big size and specific positions or morphologies. Further modifications are required to generalize this method in the future.

Regarding adverse events, the present study did not show that P-EPSS reduced the occurrence of adverse events, although the sample size may have been insufficient to detect differences between

groups because of the retrospective design. Interestingly, the selection of P-EPSS- or C-EPSS-assisted closure showed no relationship with the adverse event rate, but defects with incomplete closure and defect size larger than 3 cm were associated with worse outcomes. Cases of adverse events were successfully managed by endoscopic treatment and conservative therapy. After the treatment, the fasting period, duration of hospitalization, and total cost were similar between the two groups. Taken together, our results suggest that the P-EPSS procedure is an effective and safe method for closing the defects of difficult targets in endoscopic operations.

Our study had several limitations, including a selection bias caused by the inclusion of more cases of large defects and intraoperative perforation in the P-EPSS group; this was primarily attributable to the single-center retrospective design and should be taken into account when interpreting the findings. However, we could not eliminate selection bias. We tried to overcome these limitations by including consecutive patients during the study period. Second, all procedures were performed by expert endoscopists at a high-volume center, limiting the generalizability of the findings. We hope to design a prospective study with a different cohort to compare the criteria for choosing between P-EPSS or C-EPSS before the endoscopic manipulation. Nevertheless, despite these limitations, our study is the largest case series on the topic of EPSS-assisted endoscopic closure and the first report to present a comparison of P-EPSS and C-EPSS.

CONCLUSION

Our findings revealed that EPSS could achieve secure complete closure of mucosal defect. P-EPSS could shorten the procedure time and achieve secure complete closure of mucosal defects in clinical practice. Selection of P-EPSS was based on the defect's size, location, and the depth of the SMT. Defects with incomplete closure or size larger than 3 cm were associated with worse outcomes, rather than the selection of closure type.

ARTICLE HIGHLIGHTS

Research background

Endoscopic purse-string assisted suturing (EPSS) has proven to be an effective and safe technique for the closure of large mucosal defects. Recently, the endoscopic pre-purse-string suture (P-EPSS) procedure was invented and was widely used in clinical.

Research motivation

The novel invented P-EPSS should be analysed and compared with the conventional EPSS (C-EPSS) procedure.

Research objectives

Elucidate the outcomes of EPSS-assisted closure in different clinical situations, and evaluate the efficacy of P-EPSS.

Research methods

The study included a total of 180 patients who underwent closure assisted by EPSS between July 2014 and June 2020. The P-EPSS (n = 63) and C-EPSS (n = 117) groups were compared and the intergroup differences in aspects such as the lesion size, location, and morphology, incidence of complete closure, intraoperative perforation, and delayed adverse events were evaluated.

Research results

Patients with lesion size larger than 3 cm, lesions located at the fundus of stomach, or submucosal tumors originating from the deep mucosa were more likely to undergo P-EPSS-assisted closure. The P-EPSS group showed a significantly higher proportion of intraoperative perforation and a much shorter procedure time. But the incidence of adverse events did not differ significantly between P-EPSS and C-EPSS groups. Lesions with incomplete closure or size greater than 3 cm showed a statistical tendency to result in an increase in delayed adverse events.

Research conclusions

EPSS could achieve secure complete closure of mucosal defect. P-EPSS could shorten the procedure and yield complete closure of mucosal defects.

Research perspectives

To eliminate the selection bias, we would dedicate to design a new cohort prospective study to compare

the criteria of deciding in advance whether to use P-EPSS or C-EPSS before endoscopic manipulations.

FOOTNOTES

Author contributions: Li MM and Zhang Y contributed equally to this work; Li MM and Zhang Y drafted the manuscript and analyzed the data; Xu LM and Zhang Y designed the study and supervised its implementation; Xu LM, Zhang Y, Qu CY, and Shen F completed the endoscopic manipulations; Sun F, Huai MX, Zhang FY and Li ZH, participated in the experiments; and all authors made critical revisions and approved the final version to be published.

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Informed consent statement: Informed consent was obtained from all individual participants included in the study.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at xuleiming@xinhuamed.com.cn. Participants gave informed consent for data sharing.

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Country/Territory of origin: China

ORCID number: Ming-Ming Li 0000-0003-1753-6533; Yi Zhang 0000-0003-4208-6546; Fang Sun 0000-0003-4621-1332; Fei-Yu Zhang 0000-0002-5771-4579; Chun-Ying Qu 0000-0001-9076-081X; Feng Shen 0000-0002-8572-2074; Lei-Ming Xu 0000-0002-6735-4853.

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ORIGINAL ARTICLE

Observational Study

Trends in gastrointestinal disease hospitalizations and outcomes during the first year of the coronavirus pandemic

Ayooluwatomiwa Deborah Adekunle, Muni Rubens, Mary Sedarous, Tahniyat Tariq, Philip N Okafor

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Ayooluwatomiwa Deborah Adekunle, Division of Internal Medicine, St. Luke's Hospital, Chesterfield, MI 63017, United States

Muni Rubens, Office of Clinical Research, Miami Cancer Institute, Miami, FL 33176, United States

Mary Sedarous, Division of Internal Medicine, McMaster University, Hamilton ON L8S 4L8, Ontario, Canada

Tahniyat Tariq, Division of Gastroenterology, Stanford University School of Medicine, Redwood City, CA 94063, United States

Philip N Okafor, Division of Gastroenterology, Stanford University College of Medicine, Redwood City, CA 94063, United States

Corresponding author: Philip N Okafor, FACG, MD, Doctor, Division of Gastroenterology, Stanford University College of Medicine, 420 Broadway Street, Pavilion D, 2nd Floor, Redwood City, CA 94063, United States. philokafor@gmail.com

Abstract

BACKGROUND

The impact of the coronavirus on hospitalizations for gastrointestinal (GI) disease, particularly at a population level is understudied.

AIM

To investigate trends in hospitalizations, inpatient endoscopy resource utilization, and outcomes during the first year of the coronavirus pandemic and subsequent lockdowns.

METHODS

Using the California State Inpatient Database for 2018-2020, we explored year-to-year and 2020 month-to-month trends in hospitalizations, length of stay, and inpatient mortality (all-cause & viral pneumonia-specific) for common inpatient GI diagnoses including acute pancreatitis, diverticulitis, cholelithiasis, non-infectious gastroenteritis, upper and lower GI bleeding (LGIB), *Clostridium difficile*, viral gastroenteritis, inflammatory bowel disease, and acute cholangitis.

RESULTS

Disease-specific hospitalizations decreased for all included conditions except

nonvariceal upper GI bleeding (NVUGIB), LGIB, and ulcerative colitis (UC) (ptrend < 0.0001). All-cause inpatient mortality was higher in 2020 *vs* 2019, for acute pancreatitis (P = 0.029), diverticulitis (P = 0.04), NVUGIB (P = 0.003), and Crohn's disease (P = 0.004). In 2020, hospitalization rates were lowest in April, November, and December. There was no significant corresponding increase in inpatient mortality except in UC (ptrend = 0.048). Viral pneumonia and viral pneumonia complicated by respiratory failure increased (P < 0.001) among GI hospitalizations. Endoscopy utilization within 24 h of admission was unchanged for GI emergencies except NVUGIB (P < 0.001).

CONCLUSION

Our findings suggest that hospitalization rates for common GI conditions significantly declined in California during the COVID pandemic, particularly in April, November and December 2020. All-cause mortality was significantly higher among acute pancreatitis, diverticulitis, NVUGIB, and Crohn's disease hospitalizations. Emergency endoscopy rates were mostly comparable between 2020 and 2019.

Key Words: COVID-19; Shelter-in-place; Procedure utilization; Outcomes; Hospitalizations; Gastrointestinal diseases

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Core Tip: In the current study, we found that, in the first year of the pandemic (2020), the lowest hospitalization rates for common gastrointestinal (GI) conditions in California coincided with peaks of the pandemic in April, November, and December. Overall, there was a 15% hospitalization rate reduction for acute GI conditions in 2020 (the first year of the pandemic) compared to 2019. No significant increase in all-cause mortality for GI admissions was observed for any of the conditions studied except acute pancreatitis, diverticulitis, nonvariceal upper GI bleeding (NVUGIB) and Crohn's disease. Emergency endoscopies within the first 24 h for acute GI conditions in 2020 were comparable with previous years except for NVUGIB. Unfortunately, there was a corresponding increase in all-cause mortality for NVUGIB. We also outline and create plots of the number of admissions and associated in patient all-cause mortality by month of the year for the conditions studied.

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INTRODUCTION

The COVID-19 pandemic had an immense impact on public health globally[1,2]. The first official case in the United States was confirmed on January 20, 2020[3]. Subsequently, containment measures were implemented with the goal of curtailing the spread of the coronavirus. On March 19th, 2020, the state of California became the first in the United States to issue a mandatory stay-at-home order[4]. This was eventually followed by other states which issued similar mandates[5]. In the wake of the spreading pandemic and implementation of control measures, there was a drastic decline in healthcare resource utilization for preventive, elective, and emergency purposes[2,6-8]. Widespread delays and outright refrainment from health care seeking were observed[9], as well as the dynamic restructuring of health systems to focus on expanding capacity for COVID response and limiting COVID exposure[7,8,10,11].

Multiple studies have revealed that at the peak of the pandemic, hospitalization rates among patients with acute gastrointestinal (GI) diseases dropped precipitously[12,13]. Additionally, widespread procedural delays and an overall decrease in the performance of endoscopic procedures were observed [14-16]. Many elective and ambulatory endoscopic procedures were canceled while health systems and national societies formulated guidelines. For instance, patients hospitalized with GI bleeding were found to have more severe laboratory parameters on presentation, lower odds of inpatient endoscopy, increased length of stay, and a higher likelihood of receiving a blood transfusion than those admitted during the pre-COVID period[17].

Diagnoses such as acute pancreatitis, diverticulitis, and GI hemorrhage have historically accounted for the majority of GI admissions in the United States[18]. While it is known that there was an overall

decline in admissions for non-COVID-related diagnoses, it remains unclear how outcomes of acute GI diseases evolved through the various waves of the pandemic and phases of lockdowns[7,19]. Existing data have emerged mostly from single-institution studies[7,9,15-17,20]. These studies have suggested an increase in emergency procedures during the pandemic but have yielded mixed results regarding outcomes such as in-hospital mortality rates[17,20-22].

In this study, we describe the trends in hospitalizations, endoscopy utilization, and inpatient mortality from 2018 to 2020 for twelve of the most common inpatient GI diagnoses, using a statewide database. Findings offer insight into how outcomes evolved through the various phases of the pandemic and provide population-level data beneficial to healthcare systems and policymakers.

MATERIALS AND METHODS

Data for this study was obtained from the California Healthcare Cost and Utilization Project State Inpatient Databases (HCUP-SIDs) for the years 2018 through 2020. The SID are a set of state-specific, all-payer, administrative databases maintained by the Agency for Healthcare Research and Quality as part of the HCUP. These data are collected annually and include discharge information from over 90% of discharges from eligible hospitals. The data elements captured include demographic information such as patient age, sex, race, marital status, zip code, payer, and income quartile (based on the median income for residential zip code) as well as elements pertaining to hospitalization such as length of stay, discharge disposition, primary and secondary discharge diagnoses, procedures, total hospital charges and outcomes[23,24]. An extensive description of the SID is available elsewhere[23,24]. Based on the determination that this study did not meet the Department of Health and Human Services definition of human subject research, this study was considered exempt by the Stanford University Institutional Review Board.

Study population

The analysis cohort comprised adult (≥ 18 years old) hospitalizations for 12 acute GI conditions in California, between January 1, 2018, and December 31, 2020. We used International Classification of Diseases, Tenth Revision, Clinical Modification (ICD-10-CM) codes, similar to previous studies (see appendix) for disease identification[25]. Hospitalizations for acute pancreatitis, cholelithiasis, diverticulitis, non-infectious gastroenteritis/colitis, non-variceal upper GI bleeding, variceal upper GI bleeding, lower GI bleeding/diverticular bleeding, *Clostridium difficile*, viral gastroenteritis, Crohn's disease, ulcerative colitis (UC) were included for analysis (see appendix for the ICD code algorithms used). These diagnosis categories were chosen based on previously published population-level studies that showed them as the most prevalent inpatient GI diseases per rank ordering[26]. Observations of 10 or fewer in count were excluded from reporting per the HCUP data use agreement. All authors had access to the study data and reviewed and approved the final manuscript.

Outcome measures

The outcomes of interest included year-to-year trends from 2018 to 2019, and 2020 month-to-month variations in acute GI hospitalizations, hospital length of stay, and all-cause inpatient mortality. We also explored viral pneumonia-associated mortality outcomes from 2018 to 2020 among patients hospitalized with GI conditions to assess the burden on these patients. In addition, we analyzed patterns in endoscopy intervention for emergency GI conditions such as food impaction, foreign body ingestion, acute variceal/non-variceal hemorrhage, and acute cholangitis during the pandemic. These procedures were defined using the International Classification of Diseases, Tenth Revision, procedural coding system (ICD-10 PCS) codes similar to those used in previous studies[26,27] (please see appendix for ICD codes used). Hospitalization costs were not estimated because at the time of analysis 2020 cost-to-charge data were not available. We followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guidelines to improve the quality of reporting[28].

Statistical analysis

Descriptive statistics were used to describe demographic and clinical characteristics of GI-related hospitalizations. Frequencies and percentages were used to describe categorical variables. To compare categorical variables, we used Chi-square test. We compared demographic and clinical characteristics of GI admissions by year of hospitalizations. For selected GI conditions, we compared the total number of discharges, hospital length of stay, and in-hospital mortality by year. For selected GI procedures, we compared the total number of discharges, hospital length of stay, in-hospital mortality, number of procedures within the first 24 h of admission, and number of procedures after the first 24 h of admission. For estimating trends, we used logistic regression. Statistical significance was set at $P < 0.05$ and all tests were two-tailed. All analyses were performed using SAS version 9.4 (Cary NC, United States).

RESULTS

Baseline characteristics and hospitalization trends

Between January 1, 2018 and December 31, 2020, there were a total of 486543 hospitalizations for the 12 included acute GI conditions in California State. While annual hospitalizations were comparable between 2018 and 2019, there was a 15% hospitalization rate reduction in 2020 (173535 in 2018, 174827 in 2019, and 145545 in 2020). For all the years included in the analysis, acute cholelithiasis was the most common GI discharge diagnosis followed by acute upper GI bleeding, and then acute pancreatitis (Table 1). Disease-specific hospitalizations also significantly decreased for all conditions studied except non-variceal upper GI bleeding (NVUGIB) (P for trend = 0.853), lower GI bleeding (P for trend = 0.329), and UC (P for trend = 0.132) (Table 2). The 2020 month-to-month trend analysis revealed the lowest hospitalization rates in April, November, and December 2020, corresponding with the peaks of the pandemic (Figure 1A).

Baseline patient characteristics were similar across study years. Patients aged 45-64 years accounted for the greatest proportion of hospitalizations (33.2% in 2018, 32.7% in 2019, and 32% in 2020) while those aged ≥ 85 accounted for the smallest proportion (8.7% in 2018, 8.6% in 2019, and 8.3% in 2020) (Table 3). The majority of hospitalizations were among females (54% in 2018, 53.9% in 2019, and 52.7% in 2020) and Non-Hispanic Whites (48.3% in 2018, 46.8% in 2019, and 46.2% in 2020). Hispanics accounted for the second-highest proportion of hospitalizations (32% in 2018, 32.5% in 2019, and 33% in 2020) while other racial groups accounted for a minority of hospitalizations across all 3 study years (Table 3).

Mortality trends

For the entire cohort of GI hospitalizations, all-cause inpatient mortality was higher in 2020 compared to 2019 (1.2% *vs* 1.0%, $P < 0.001$). However, when individual diagnoses were examined, for most of the diseases, all-cause inpatient mortality was no different in 2020 compared to 2019 except among patients hospitalized with acute pancreatitis (0.6% *vs* 0.5%, $P = 0.029$), diverticulitis (0.6% *vs* 0.4%, $P = 0.04$), NVUGIB (2.1% *vs* 1.8%, $P = 0.003$), and Crohn's disease (2.2% *vs* 1.7%, $P = 0.004$) (Table 2).

To determine the trends in all-cause GI inpatient mortality, a 2020 month-to-month analysis was performed that did not reveal any significant variations except for UC hospitalizations where the highest inpatient mortality rate was observed in December 2020 (5%, P for trend = 0.048) (Table 2). We also examined month-to-month (Supplementary Figure 1), and year-to-year changes in viral pneumonia admissions among patients with the aforementioned GI diagnoses. We observed a significant increase in viral pneumonia hospitalizations among the entire cohort and also a significant increase in viral pneumonia-specific mortality from 2018 to 2020 (Table 3). In the absence of a dedicated ICD-10 code for COVID-19 in the years preceding the pandemic, we could not directly compare rates. We did however examine COVID-19-specific mortality rates for the year 2020 and found a mortality rate of 0.08%.

Endoscopy utilization

We examined patterns of endoscopy utilization including upper endoscopy, colonoscopy, flexible sigmoidoscopy, and endoscopic retrograde cholangiopancreatography (ERCP) in California between 2018 and 2020 for GI emergencies (Figure 1B). Except upper endoscopy for acute variceal hemorrhage which significantly reduced in 2020 (744 *vs* 690, $P = 0.007$), likely as a result of overall reduced hospitalization rates, there was no significant difference in inpatient endoscopic intervention rates in 2020 compared to 2019 (Table 4). Trend analysis showed that mortality for GI bleed emergencies was comparable for acute variceal and lower GI bleeding in the pre-pandemic and pandemic eras. However, in 2020, there was a significant rise in all-cause mortality among patients hospitalized with acute nonvariceal upper GI bleeding.

Sensitivity analyses demonstrated that for patients with acute nonvariceal bleeding, a significantly lower proportion received upper endoscopic intervention within the first 24 h of admission in 2020 compared to 2019 (30.3% *vs* 32.5%, $P < 0.001$), while the proportions were unchanged compared to previous years for patients hospitalized with acute variceal bleeding and lower GI bleeding (Table 4). ERCP utilization for acute cholangitis, intervention within the first 24 h (24.9% *vs* 27%, $P = 0.700$) and mortality from acute cholangitis (0.6% *vs* 0.4%. $P = 0.950$) did not significantly differ between all 3 years (Table 4). A graphical representation of monthly trends in endoscopy interventions and mortality in 2020 is shown in Supplementary Figure 2.

DISCUSSION

In this population-level study, we evaluated trends and outcomes of hospitalizations for acute GI conditions in 2020, the first year of the COVID pandemic in California, using a statewide database. Using the 2018 and 2019 data as a comparator group, we found significantly lower rates of hospitalization for most acute GI conditions in 2020 compared to previous years. We also found concomitant increases in all-cause inpatient mortality for four of the diagnoses studied including acute pancreatitis,

Table 1 Trends in discharges, hospital stay in days, and mortality for selected gastrointestinal diseases in California, 2018-2020

GI conditions	2018	2019	2020	Relative change	*P* for trend
Acute pancreatitis					
Total number of discharges, *n* (%)	26771 (0.8)	26675 (0.8)	23828 (0.8)	-11.0	0.028
Total hospital stays (in thousands) days	109.6	107.6	99.4	-9.3	0.118
Mortality, *n* (%)	124 (0.5)	134 (0.5)	144 (0.6)	16.1	0.029
Cholelithiasis					
Total number of discharges	39473 (1.2)	39710 (1.3)	33215 (1.2)	-15.9	< 0.001
Total hospital stays (in thousands) days	129.1	130.5	107.4	-16.8	0.152
Mortality, *n* (%)	96 (0.2)	94 (0.2)	81 (0.2)	-15.6	0.994
Diverticulitis					
Total number of discharges	16899 (0.5)	16992 (0.5)	13855 (0.5)	-18.0	< 0.001
Total hospital stays (in thousands) days	75.7	76.0	61.5	-18.8	0.495
Mortality, *n* (%)	77 (0.5)	67 (0.4)	87 (0.6)	13.0	0.042
Noninfectious gastroenteritis/colitis					
Total number of discharges	9376 (0.3)	9236 (0.3)	6734 (0.2)	-28.2	< 0.001
Total hospital stays (in thousands) days	27.3	27.8	21.3	-22.0	< 0.001
Mortality, *n* (%)	31 (0.3)	35 (0.4)	26 (0.4)	-16.1	0.543
Nonvariceal upper GI bleeding					
Total number of discharges	32207 (1.0)	33361 (1.1)	29155 (1.0)	-9.5	0.853
Total hospital stays (in thousands) days	125.6	132.6	116.9	-6.9	0.002
Mortality, *n* (%)	581 (1.8)	604 (1.8)	623 (2.1)	7.2	0.003
Variceal upper GI bleeding					
Total number of discharges	1007 (0.0)	880 (0.0)	808 (0.0)	-19.8	0.006
Total hospital stays (in thousands) days	4.0	3.7	3.2	-20.0	0.635
Mortality, *n* (%)	35 (3.5)	38 (4.3)	39 (4.8)	11.4	0.149
Lower GI bleeding and diverticular bleeding					
Total number of discharges	11549 (0.4)	11914 (0.4)	10613 (0.4)	-8.1	0.329
Total hospital stays (in thousands) days	43.4	44.2	40.0	-7.8	0.783
Mortality, *n* (%)	132 (1.1)	127 (1.1)	100 (0.9)	-24.2	0.145
Clostridium difficile					
Total number of discharges	1552 (0.0)	1457 (0.0)	963 (0.0)	-38.0	< 0.001
Total hospital stays (in thousands) days	9.8	9.3	6.3	-35.7	0.532
Mortality, *n* (%)	17 (1.1)	20 (1.4)	15 (1.6)	-11.8	0.308
Viral gastroenteritis					
Total number of discharges	6005 (0.2)	5652 (0.2)	3794 (0.1)	-36.8	< 0.001
Total hospital stays (in thousands) days	17.0	16.0	11.6	-31.8	< 0.001
Mortality, *n* (%)	15 (0.2)	19 (0.3)	18 (0.5)	20.0	0.066
Crohn's disease					
Total number of discharges	14273 (0.5)	14510 (0.5)	12583 (0.4)	-11.8	0.021
Total hospital stays (in thousands) days	76.6	77.5	70.1	-8.5	0.060
Mortality, *n* (%)	245 (1.7)	242 (1.7)	276 (2.2)	12.7	0.004
Ulcerative colitis					

Total number of discharges	12805 (0.4)	12919 (0.4)	11848 (0.4)	-7.5	0.132
Total hospital stays (in thousands) days	74.7	75.5	70.5	-5.6	0.361

GI: Gastrointestinal.

diverticulitis, NVUGIB, and Crohn's disease in 2020 compared to 2019. In addition, a 2020 month-to-month hospitalization trends analysis demonstrated the lowest hospitalization rates in April, November, and December of 2020.

The observed decline in hospitalizations for acute GI conditions is consistent with findings from previously published studies and likely reflects an interplay of factors during the COVID pandemic[12,13,29]. First, it has been suggested that people were willing to delay much-needed healthcare out of fear of potential exposure to COVID-19[9,11,29,30]. Additionally, the shelter-in-place measures in the spring of 2020 along with the restructuring of healthcare services necessitated by the pandemic are believed to have further reduced healthcare utilization for non-COVID indications[7,29].

The April nadir occurred after the implementation of multiple measures including the cancellation of all large events and the issuance of a stay-at-home order in March, by the California state government [13]. After the release of an initial reopening plan on April 28th, 2020 by the governor of California; and during the implementation of the early phases of the plan (in the summer months), we observed an uptick in hospitalization rates[31]. However, these never returned to pre-pandemic numbers. Coinciding with the reintroduction of restrictions later in the year, total hospitalization numbers for acute GI conditions plunged again. These lower hospitalization numbers continued through December as California and the rest of the United States battled another wave of the coronavirus pandemic[31].

Regarding GI outcomes with the fluctuations in hospitalization rates, fortunately, for most of the acute GI conditions studied, there was no significant increase in all-cause inpatient mortality, except for acute pancreatitis, diverticulitis, NVUGIB, and Crohn's disease admissions. Although we could not determine the time of symptom onset before admission, it is possible that previously reported delays in presentation coupled with higher acuity on presentation could have contributed to the observed increases in mortality for these conditions. A previous study of acute myocardial infarctions revealed significant delays in presentation and higher mortality in the setting of the pandemic[9]. Additionally, a collaborative report from Harvard T.H. Chan School of Public Health, the Robert Wood Johnson Foundation, and National Public Radio revealed that 2 in 10 adults had reported having household members who had delayed care for a serious medical condition in the setting of the pandemic. Furthermore, over 50% of those reporting delayed care had also reported experiencing resultant adverse effects[29]. The delayed presentation has been correlated with worse health outcomes[32].

Multiple studies reported increases in alcohol intake during the pandemic which could potentially explain the worse outcomes seen in acute pancreatitis hospitalizations[33,34]. However, we could not reliably ascertain the etiology of acute pancreatitis given the claims-based nature of the databases. In addition, we observed increased mortality among patients with Crohn's disease, but it remains unclear if the immunosuppression associated with the treatment of this inflammatory bowel disease increases the risk of COVID-related morbidity and mortality and/or if the excess mortality was attributable to COVID.

Although widespread procedural delays have been reported in the setting of the pandemic, our analysis of emergency procedural utilization revealed comparable rates of endoscopies for emergency GI conditions in 2020 with previous years, except when it came to NVUGIB where we observed a significant reduction in the proportion of patients receiving upper endoscopy within the first 24 h of presentation[14-16]. While this suggests that procedural delays could have played a role in the increase in NVUGIB-related inpatient mortality observed in 2020, it is difficult to attribute our findings solely to procedural delays as delays in presentation and higher acuity on presentation could have also played a role[9,29]. Nonetheless, current guidelines do recommend endoscopy within 24 h of presentation, and delays in endoscopy have been correlated with adverse outcomes in NVUGIB[35,36]. It is reassuring to note that outcomes of other GI emergencies such as acute variceal upper GI bleeding, lower GI bleeding, and acute cholangitis was not adversely impacted during the COVID pandemic. Although for these conditions, no reduction in emergency procedural utilization was noted.

The current study has multiple strengths. First, the use of a large, all-payer, statewide database allowed us to capture the impact of the pandemic at a large population level factoring in a diverse group of patients with different payer types. Additionally, our analyses of month-to-month trends for the year 2020, allowed us to evaluate trends in outcomes of interest in the light of the trajectory of the pandemic and our findings were reasonable as the lowest hospitalization numbers matched the phases of the documented lockdowns. We were also able to utilize ICD-10 codes and cohort identification algorithms that may reduce the risk of misclassification bias. Furthermore, we were able to compare 2020 data with the 2018 and 2019 SID data which provided baseline, pre-pandemic rates.

This study, however, has its limitations. First, given the widely varying approaches to the pandemic taken by individual states in the United States, generalizability might be limited. However, considering

Table 2 2020 month-to-month disease-specific mortality trends, *n* (%)

	January, 2020	February, 2020	March, 2020	April, 2020	May, 2020	June, 2020	July, 2020	August, 2020	September, 2020	October, 2020	November, 2020	December, 2020	Relative change	*P* for trend
Acute pancreatitis														
Total number of discharges	2163 (0.8)	1935 (0.8)	1956 (0.9)	1642 (0.9)	2002 (0.9)	2092 (0.9)	2063 (0.8)	2077 (0.9)	2106 (0.9)	2096 (0.8)	1913 (0.8)	1783 (0.7)	-17.6%	< 0.0001
Mortality	21 (1.0)	12 (0.6)	16 (0.8)	8 (0.5)	6 (0.3)	12 (0.6)	11 (0.5)	14 (0.7)	12 (0.6)	8 (0.4)	10 (0.5)	14 (0.8)	-33.3%	0.211
Cholelithiasis														
Total number of discharges	3170 (1.1)	2926 (1.1)	2437 (1.1)	2050 (1.1)	2743 (1.2)	2831 (1.2)	2759 (1.1)	2858 (1.2)	3112 (1.3)	3137 (1.3)	2864 (1.2)	2328 (1.0)	-26.6%	< 0.0001
Mortality	6 (0.2)	9 (0.3)	2 (0.1)	4 (0.2)	7 (0.3)	4 (0.1)	3 (0.1)	7 (0.2)	14 (0.4)	7 (0.2)	8 (0.3)	10 (0.4)	66.7%	0.060
Diverticulitis														
Total number of discharges	1349 (0.5)	1315 (0.5)	1067 (0.5)	746 (0.4)	1050 (0.5)	1141 (0.5)	1183 (0.5)	1302 (0.5)	1247 (0.5)	1351 (0.5)	1161 (0.5)	943 (0.4)	-30.1%	0.0004
Mortality	9 (0.7)	11 (0.8)	11 (1.0)	6 (0.8)	3 (0.3)	3 (0.3)	4 (0.3)	9 (0.7)	10 (0.8)	7 (0.5)	7(0.6)	7 (0.7)	-22.2%	0.501
Noninfectious gastroenteritis/colitis														
Total number of discharges	720 (0.3)	643 (0.3)	538 (0.2)	426 (0.2)	539 (0.2)	535 (0.2)	554 (0.2)	560 (0.2)	641 (0.3)	670 (0.3)	510 (0.2)	398 (0.2)	-44.7%	< 0.0001
Mortality	2 (0.3)	2 (0.3)	3 (0.6)	2 (0.5)	2 (0.4)	2 (0.4)	3 (0.5)	2 (0.4)	2 (0.3)	2 (0.3)	1 (0.2)	3 (0.8)	50.0%	0.859
Nonvariceal upper GI bleeding														
Total number of discharges	2971 (1.1)	2692 (1.1)	2458 (1.1)	1960 (1.0)	2361 (1.1)	2440 (1.0)	2451 (1.0)	2325 (1.0)	2428 (1.0)	2396 (1.0)	2450 (1.0)	2223 (0.9)	-25.2%	<.0001
Mortality	68 (2.3)	61 (2.3)	52 (2.1)	55 (2.8)	48 (2.0)	40 (1.6)	49 (2.0)	35 (1.5)	46 (1.9)	47 (2.0)	72 (2.9)	50 (2.2)	-26.5%	0.882
Variceal upper GI bleeding														
Total number of discharges	80 (0.0)	59 (0.0)	71 (0.0)	53 (0.0)	55 (0.0)	74 (0.0)	65 (0.0)	64 (0.0)	73 (0.0)	57 (0.0)	74 (0.0)	83 (0.0)	3.8%	0.042
Mortality	2 (2.5)	2 (3.4)	1 (1.4)	1 (1.9)	1 (1.8)	6 (8.1)	5 (7.7)	4 (6.3)	6 (8.2)	2 (3.5)	2 (2.7)	7 (8.4)	250.0%	0.050
Lower GI bleeding and diverticular bleeding														
Total number of discharges	1039 (0.4)	943 (0.4)	884 (0.4)	743 (0.4)	843 (0.4)	834 (0.4)	835 (0.3)	906 (0.4)	936 (0.4)	916 (0.4)	921 (0.4)	813 (0.3)	-21.8	0.231
Mortality	13 (1.3)	6 (0.6)	12 (1.4)	5 (0.7)	9 (1.1)	6 (0.7)	7 (0.8)	9 (1.0)	7 (0.7)	7 (0.8)	8 (0.9)	11 (1.4)	-15.4	0.822

Clostridium difficile														
Total number of discharges	96 (0.0)	98 (0.0)	85 (0.0)	63 (0.0)	67 (0.0)	81 (0.0)	82 (0.0)	80 (0.0)	89 (0.0)	95 (0.0)	70 (0.0)	57 (0.0)	-40.6	0.131
Mortality	2 (2.1)	2 (2.0)	4 (4.7)	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.4)	4 (5.0)	0 (0.0)	0 (0.0)	1 (1.4)	0 (0.0)	-100.0	0.157
Viral gastroenteritis														
Total number of discharges	468 (0.2)	395 (0.2)	366 (0.2)	248 (0.1)	263 (0.1)	296 (0.1)	288 (0.1)	325 (0.1)	325 (0.1)	327 (0.1)	296 (0.1)	197 (0.1)	-57.9%	0.536
Mortality	4 (0.9)	0 (0.0)	2 (0.5)	2 (0.8)	2 (0.8)	3 (1.0)	0 (0.0)	0 (0.0)	2 (0.6)	1 (0.3)	2 (0.7)	0 (0.0)	-100.0%	0.402
Crohn's disease														
Total number of discharges	1262 (0.5)	1137 (0.4)	977 (0.4)	817 (0.4)	998 (0.5)	997 (0.4)	1076 (0.4)	1102 (0.5)	1120 (0.5)	1078 (0.4)	1040 (0.4)	979 (0.4)	-22.4%	0.035
Mortality	29 (2.3)	26 (2.3)	18 (1.8)	25 (3.1)	16 (1.6)	25 (2.5)	16 (1.5)	20 (1.8)	25 (2.2)	22 (2.0)	23 (2.2)	31 (3.2)	6.9%	0.627
Ulcerative colitis														
Total number of discharges	1109 (0.4)	1038 (0.4)	917 (0.4)	787 (0.4)	953 (0.4)	984 (0.4)	1039 (0.4)	973 (0.4)	1068 (0.4)	1081 (0.4)	970 (0.4)	929 (0.4)	-16.2%	0.0005
Mortality	31 (2.8)	32 (3.1)	19 (2.1)	28 (3.6)	19 (2.0)	23 (2.3)	22 (2.1)	23 (2.4)	26 (2.4)	27 (2.5)	35 (3.6)	46 (5.0)	48.4%	0.048
Acute cholangitis														
Total number of discharges	121 (0.0)	107 (0.0)	111 (0.0)	74 (0.0)	108 (0.0)	103 (0.0)	101 (0.0)	126 (0.1)	128 (0.1)	161 (0.1)	118 (0.0)	108 (0.0)	-10.7%	0.032
Mortality	1 (0.8)	0 (0.0)	0 (0.0)	2 (2.7)	0 (0.0)	1 (1.0)	1 (1.0)	0 (0.0)	1 (0.8)	1 (0.6)	0 (0.0)	2 (1.9)	100.0%	0.671

GI: Gastrointestinal.

that our study corroborates findings from previous studies, it is unlikely that the observed patterns are only limited to California[12,13]. Another limitation is that we could not explore time to presentation and therefore cannot definitively conclude that delays in presentation contributed to the observed increases in mortality. Our study also did not explore out-of-hospital mortality, making it possible that the overall pandemic-related excess mortality is higher than observed in our study.

Also, the timeframe of the data did not include 2021. Consequently, we were unable to explore the evolution of outcomes after the initiation of widespread vaccination, beginning in late 2020, as well as in the light of the Delta and Omicron variant-related surges. It is also not possible to ascertain if the trends continued into 2021. Finally, we have to emphasize the possibility of misclassification bias given our use of an administrative dataset.

Table 3 Demographic and clinical characteristics of select gastrointestinal hospitalizations in California, 2018-2020, *n* (%)

Variables	2018	2019	2020	*P* value
Age, yr				< 0.001
18-44	47019 (27.7)	46521 (27.2)	40924 (28.1)	
45-64	56369 (33.2)	5598 (32.7)	46629 (32.0)	
65-84	51757 (30.5)	53860 (31.5)	45944 (31.6)	
≥ 85	14693 (8.7)	14806 (8.6)	12062 (8.3)	
Sex				< 0.001
Male	78081 (46.0)	78928 (46.1)	68873 (47.3)	
Female	91745 (54.0)	92239 (53.9)	76677 (52.7)	
Race				< 0.001
White	81404 (48.3)	79531 (46.8)	66613 (46.2)	
African American	13080 (7.8)	13016 (7.7)	11219 (7.8)	
Hispanic	53962 (32.0)	55153 (32.5)	47629 (33.0)	
Asian Pacific Islander	13422 (8.0)	13894 (8.2)	11375 (7.9)	
Native American	555 (0.3)	835 (0.5)	485 (0.3)	
Other	6286 (3.7)	7336 (4.3)	6947 (4.8)	
Payer				< 0.001
Medicare	68639 (40.4)	70861 (41.4)	59701 (41.0)	
Medicaid	48581 (28.6)	48048 (28.1)	42046 (28.9)	
Private	45612 (26.9)	44389 (25.9)	37733 (25.9)	
Self-pay	3492 (2.1)	3864 (2.3)	2948 (2.0)	
No charge	204 (0.1)	272 (0.2)	170 (0.1)	
Other	3254 (1.9)	3707 (2.2)	2931 (2.0)	
Elixhauser comorbidity index				< 0.001
0	28927 (17.0)	28648 (16.7)	23244 (16.0)	
1	31228 (18.4)	31049 (18.1)	26001 (17.9)	
2	28036 (16.5)	28078 (16.4)	24118 (16.6)	
≥ 3	81647 (48.1)	83401 (48.7)	72196 (49.6)	
Bacterial pneumonia	4232 (2.5)	4285 (2.5)	3856 (2.6)	0.008
Bacterial pneumonia/respiratory failure specific mortality	248 (0.1)	271 (0.2)	275 (0.2)	0.009
Viral pneumonia	76 (0.0)	82 (0.0)	724 (0.5)	< 0.001
Viral pneumonia/respiratory failure specific mortality	6 (0.0)	5 (0.0)	80 (0.1)	< 0.001
COVID-19 specific mortality	-	-	123 (0.08)	< 0.001
All-cause mortality	1667 (1.0)	1644 (1.0)	1723 (1.2)	< 0.001

COVID-19: Coronavirus disease 2019.

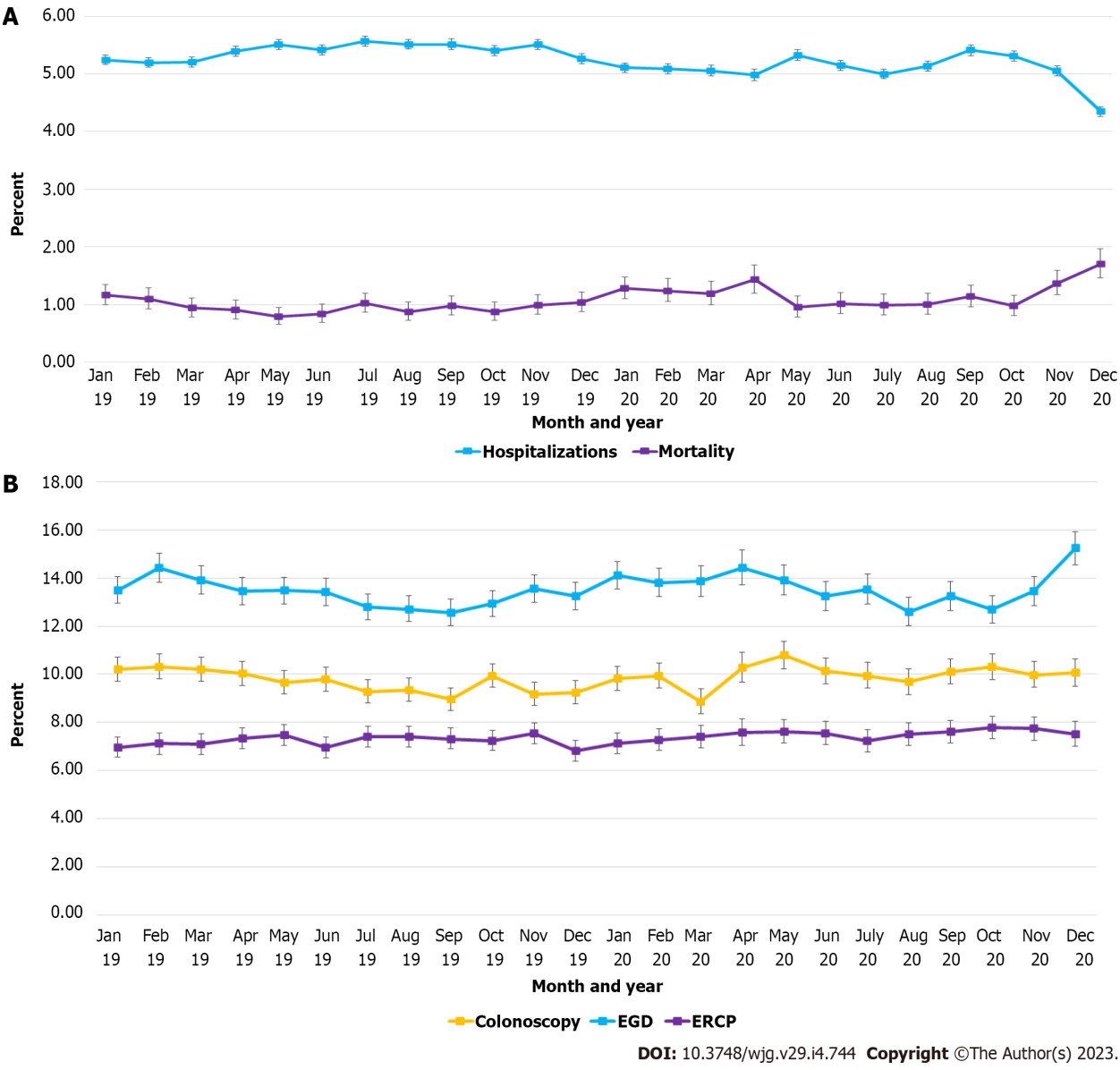
CONCLUSION

In conclusion, our findings suggest that during the first year of the COVID pandemic, there was a significant decline in hospitalization rates for common GI conditions in California, particularly in the months of April, November, and December 2020. Reassuringly, 2020 emergency endoscopy rates were mostly comparable with 2019 rates except when it came to emergency endoscopy for NVUGIB. These findings suggest that inpatient healthcare delivery for most patients with acute GI conditions remained largely unchanged during the COVID-19 pandemic. They do however reveal that patients hospitalized with acute pancreatitis, diverticulitis, nonvariceal upper GI bleeding and Crohn's disease experienced

Table 4 Trends in discharges, mortality rate, and endoscopy utilization for gastrointestinal emergencies discharges in California, 2018-2020, *n* (%)

	2018	2019	2020	Relative change	*P* for trend
ANVH (upper endoscopy)					
Total number of discharges	15310 (0.5)	15942 (0.5)	13622 (0.5)	-11.0%	0.121
Total hospital stays (in thousands) days	65.0	69.2	60.2	-7.4%	0.002
Mortality	321 (2.1)	335 (2.1)	346 (2.5)	7.8%	0.012
Procedure within 24 h	5005 (32.7)	5185 (32.5)	4132 (30.3)	-17.4%	< 0.001
AVH (upper endoscopy)					
Total number of discharges	867 (0.0)	744 (0.0)	690 (0.0)	-20.4%	0.007
Total hospital stays (in thousands) days	3.6	3.2	2.8	-22.2%	0.697
Mortality	26 (3.0)	22 (3.0)	26 (3.8)	0.0%	0.414
Procedure within 24 h	348 (40.1)	327 (44.0)	283 (41.0)	-18.7%	0.653
LGIB (lower endoscopy)					
Total number of discharges	5524 (0.2)	5722 (0.2)	4973 (0.2)	-10.0%	0.724
Total hospital stays (in thousands) days	21.7	22.1	19.9	-8.3%	0.436
Mortality	29 (0.5)	35 (0.6)	17 (0.3)	-41.4%	0.201
Procedure within 24 h	859 (15.6)	975 (17.0)	793 (15.9)	-7.7%	0.534
Acute cholangitis (ERCP)					
Total number of discharges	1038 (0.0)	962 (0.0)	882 (0.0)	-15.0%	0.137
Total hospital stays (in thousands) days	4.3	4.4	3.7	-14.0%	0.505
Mortality	6 (0.6)	4 (0.4)	5 (0.6)	-16.7%	0.952
Procedure within 24 h	268 (25.8)	260 (27.0)	220 (24.9)	-17.9%	0.695
Food impaction (EGD/upper endoscopy)					
Total number of discharges	127 (0.0)	143 (0.0)	118 (0.0)	-7.1%	0.834
Total hospital stays (in thousands) days	0.6	0.6	0.6	0.0%	0.849
Mortality	4 (3.1)	3 (2.1)	0 (0.0)	-100.0%	0.086
Procedure within 24 h	73 (57.5)	70 (49.0)	60 (50.8)	-17.8%	0.289
Foreign body (EGD)					
Total number of discharges	21 (0.0)	17 (0.0)	20 (0.0)	-4.8%	0.891
Mortality	1 (4.8)	2 (11.8)	0 (0.0)	-100.0%	0.512
Procedure within 24 h	7 (33.3)	7 (41.2)	12 (60.0)	71.4%	0.091
SBP-paracentesis					
Total number of discharges	3254 (0.1)	3486 (0.1)	3344 (0.1)	2.8%	< 0.001
Total hospital stays (in thousands) days	32.4	35.3	33.8	4.3%	0.594
Mortality	621 (19.1)	631 (18.1)	592 (17.7)	-4.7%	0.147
Procedure within 24 h	928 (28.5)	1044 (30.0)	1022 (30.6)	10.1%	0.065

ANVH: Acute nonvariceal hemorrhage; AVH: Acute viral hepatitis; LGIB: Lower gastrointestinal bleeding; ERCP: Endoscopic retrograde cholangiopancreatography; EGD: Esophagogastroduodenoscopy; SBP: Spontaneous bacterial peritonitis.



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Figure 1 Monthly trends. A: Monthly trends in hospitalization of selected gastrointestinal conditions and in-hospital mortality rates from January 2019 to December 2020; B: Monthly trends in endoscopy procedures among hospitalizations for selected gastrointestinal conditions from January 2019 to December 2020. EGD: Esophagogastroduodenoscopy; ERCP: Endoscopic retrograde cholangiopancreatography.

higher all-cause inpatient mortality during the pandemic and highlight that further research is needed to elucidate the disease-specific and system-based risk factors for the increase in mortality observed in these conditions.

ARTICLE HIGHLIGHTS

Research background

Healthcare resource utilization declined during the coronavirus pandemic. How this impacted gastrointestinal (GI) disease hospitalizations is not fully understood. We sought to investigate trends in hospitalizations, inpatient endoscopy utilization and outcomes during the first year of the pandemic and lockdowns.

Research motivation

The need for a population level understanding of the impact of the coronavirus pandemic on the outcomes of patients hospitalized with GI diseases.

Research objectives
To investigate trends in hospitalizations, inpatient endoscopy utilization and outcomes during the first year of the pandemic and lockdowns.

Research methods
Using the California State Inpatient Database for 2018-2020, we explored year-to-year and 2020 month-to-month trends in hospitalizations, length of stay, and inpatient mortality (all-cause & viral pneumonia-specific) for common inpatient GI diagnoses including acute pancreatitis, diverticulitis, cholelithiasis, noninfectious gastroenteritis, upper and lower GI bleeding (LGIB), *Clostridium difficile*, viral gastroenteritis, inflammatory bowel disease, and acute cholangitis, using regression analyses. We also investigated endoscopy utilization for GI emergencies.

Research results
Disease-specific hospitalizations decreased for all included conditions except nonvariceal upper GI bleeding (NVUGIB), LGIB, and ulcerative colitis (UC) (ptrend < 0.0001). All-cause inpatient mortality was higher in 2020 compared to 2019, for acute pancreatitis (P = 0.029), diverticulitis (P = 0.04), NVUGIB (P = 0.003), and Crohn's disease (P = 0.004). In 2020, hospitalization rates were lowest in April, November, and December. There was no significant corresponding increase in inpatient mortality except in UC (ptrend = 0.048). Endoscopy utilization within 24 h of admission was unchanged for GI emergencies except NVUGIB in which it was lower (P < 0.001).

Research conclusions
Our findings suggest that hospitalization rates for common GI conditions significantly declined in California during the COVID pandemic, particularly in April, November and December 2020. All-cause mortality was significantly higher among acute pancreatitis, diverticulitis, NVUGIB, and Crohn's disease hospitalizations. Emergency endoscopy rates were mostly comparable between 2020 and 2019.

Research perspectives
We observed that patients hospitalized with acute pancreatitis, diverticulitis, nonvariceal upper GI bleeding and Crohn's disease experienced higher all-cause inpatient mortality during the pandemic. Further research is needed to elucidate the disease-specific and system-based risk factors for the increase in mortality observed in these conditions.

FOOTNOTES

Author contributions: Okafor PN and Adekunle AD conceived and designed the study; Rubens M performed the statistical analysis; Adekunle AD, Rubens M, Sedarous M, and Okafor PN wrote and critically reviewed the manuscript; all authors reviewed and approved the manuscript.

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Country/Territory of origin: United States

ORCID number: Philip N Okafor 0000-0003-1695-7491.

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CASE REPORT

Pulmonary cryptococcosis after immunomodulator treatment in patients with Crohn's disease: Three case reports

Yan-Fei Fang, Xiang-Han Cao, Ling-Ya Yao, Qian Cao

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Yan-Fei Fang, Xiang-Han Cao, Ling-Ya Yao, Qian Cao, Department of Gastroenterology, Inflammatory Bowel Disease Center, Sir Run Shaw Hospital, College of Medicine Zhejiang University, Hangzhou 310016, Zhejiang Province, China

Corresponding author: Qian Cao, PhD, Chief Doctor, Chief Physician, Department of Gastroenterology, Inflammatory Bowel Disease Center, Sir Run Shaw Hospital, College of Medicine Zhejiang University, No. 3 East Qingchun Road, Hangzhou 310016, Zhejiang Province, China. caoq@zju.edu.cn

Abstract

BACKGROUND

Corticosteroids and anti-tumor necrosis factor α mAbs are widely used to treat Crohn's disease (CD). However, one disadvantage of this treatment is impairment of normal immune function, leading to an increased risk of infection. Cryptococcus infection is an opportunistic infection that occurs mainly in immunocompromised patients and poses a significant diagnostic challenge in patients with CD.

CASE SUMMARY

Here, we report three cases of pulmonary cryptococcosis in patients with CD after receiving immunomodulatory treatment. The patients presented with no or mild respiratory symptoms. Chest computed tomography scans revealed pulmonary nodules in the unilateral or bilateral lobes. Diagnoses were made using pathological examination and metagenomic sequencing. The patients were treated with fluconazole 400 mg once daily for 1 to 6 mo, and symptoms were resolved. Literature searches were conducted in PubMed, Web of Science, and Embase to retrieve previously reported cases and summarize patient characteristics.

CONCLUSION

The incidence of cryptococcus infection has increased along with immunomodulator use. Clinical vigilance is required for early identification and standardized treatment.

Key Words: Crohn's disease; Immunomodulator; Infliximab; Opportunistic infections; Pulmonary cryptococcosis; Case report

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Core Tip: Corticosteroids and anti-tumor necrosis factor α mAbs are commonly used to treat Crohn's disease (CD). However, they may also contribute to an increased risk of opportunistic infections. In this article, we report three cases of pulmonary cryptococcosis in patients with CD after receiving immunomodulatory treatment. Pathogen identification mainly depends on pathological examination findings, but metagenomics can serve as an alternative tool. Patients with timely diagnosis generally have a good prognosis, but clinical alerts should be raised in those who are elderly and have comorbidities and dissemination phenotype.

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INTRODUCTION

Crohn's disease (CD), a chronic inflammatory disease that may involve any part of the digestive tract, is characterized by periodic clinical relapse and remission[1]. Treatment medications include mesalamine, corticosteroids, small-molecule immunosuppressants, and biologics such as mAbs[2]. However, these drugs also affect normal immune function and may contribute to an increased risk of opportunistic infections.

Pulmonary cryptococcosis is a type of subacute or chronic fungal infection caused by the genus *Cryptococcus*, and is found mostly in immunocompromised patients. It usually presents with isolated pulmonary granulomatous lesions but can also disseminate to other organ systems[3]. Here, we report three cases of pulmonary cryptococcosis in patients with CD who received immunomodulatory treatment, and review previously reported cases. Literature searches were conducted in PubMed, Web of Science and Embase databases using the keywords "Crohn's disease", "pulmonary cryptococcosis", and "cryptococcus". The search was restricted to full-text journal articles and conference abstracts published in English between January 1980 and May 2022. The bibliographies of the included articles were manually reviewed.

CASE PRESENTATION

Chief complaints

Case 1: A 65-year-old man was admitted to a local hospital for recurrent abdominal distension and pain 10 years ago.

Case 2: A 20-year-old man was admitted to our hospital for perianal pain with fever 5 years ago.

Case 3: A 59-year-old man was admitted to a local hospital for recurrent abdominal pain and diarrhea 13 years ago.

History of present illness

Case 1: The patient was referred to our hospital and diagnosed with CD. Before treatment initiation, chest computed tomography (CT) showed small nodules in the bilateral upper lobes with a benign tendency, pulmonary sac in the right upper lobe, and fibrous foci in the right middle lobe and bilateral lower lobes. The patient received four doses of infliximab (IFX; 300 mg) and achieved clinical remission.

Case 2: The patient was diagnosed with CD and was treated with IFX for 2 years (22 doses; 300 mg), which was discontinued due to secondary loss of response. He complained of night sweats and right-sided chest pain during deep breathing.

Case 3: The patient was diagnosed with CD 5 years ago and had been treated with methylprednisolone (MP; 0.75 mg/kg) for 3 years. He complained of productive cough for 1 mo.

Laboratory examinations

Case 2: The carbohydrate antigen 125 (CA-125) level was 54.2 U/mL. The G test, galactomannan (GM) test, and sputum fungal test results were negative.

Case 3: Vitamin B12 and folic acid levels were normal. Hepatitis B virus surface, core and E antibodies, antinuclear antibodies, vasculitis antibodies, sputum acid-fast (AF) staining, bronchoscopic brush

cytology, and bronchoalveolar lavage fluid (BALF) cytopathology showed negative findings.

Other laboratory examination results are summarized in Table 1.

Imaging examinations

Case 1: A repeat CT scan after the fourth dose of IFX revealed an increased number of bilateral nodules and a large nodule in the left upper lobe with possible inflammation (Fig. 1A). Electromagnetic navigation bronchoscopy revealed a clear trachea and no neoplasm in the principal and segmental bronchi. A biopsy was performed in the anterior segment of the left upper lobe. Fibrinobronchoscopic AF staining, fungus, culture, brush cytology, BALF Gram staining, exfoliative cytology, and X-PERT assay (simultaneous detection of *Mycobacterium tuberculosis* and resistance to rifampin) results were negative. Biopsy revealed focal granuloma formation in the alveolar tissues (Figure 1B). Periodic acid-Schiff (PAS) staining and AF staining were negative; however, periodic acid-silver methenamine (PASM) staining revealed suspicious positive bodies (Figure 1C).

Case 2: Chest CT revealed patchy lesions in the right upper lobe and right interlobar fissure, and multiple bilateral nodules with inflammatory propensity (Figure 2A). Bronchoscopy revealed clear segmental bronchi with no apparent stenosis or neoplasm in the lumen. Fibrinobronchoscopy was performed, and the BALF Aspergillus GM level was 0.840 μg/L. AF staining, fungus, brush cytology, and culture results were negative. BALF was sent for metagenomic sequencing and Cryptococcus was detected.

Case 3: Chest CT tomography revealed nodules in the posterior segment of the right upper lobe (Figure 3A). Tumor presence could not be ruled out. Bronchoscopy revealed no neoplasm in bilateral bronchi. Considering the difficulty of bronchoscopic biopsy, thoracoscopic lobectomy of the posterior segment of the right upper lobe was performed. Postoperative pathology revealed negative AF staining results but positive PAS and PASM staining results (Figure 3B and C). Tuberculosis- DNA (TB-DNA) test was negative.

FINAL DIAGNOSIS

Case 1-3: A diagnosis of CD combined with pulmonary cryptococcosis was confirmed.

TREATMENT

Case 1: IFX was discontinued, and the patient was administered fluconazole (FLCZ) 400 mg once daily for 6 mo.

Case 2: Medications for CD were discontinued. The patient was given FLCZ 400 mg once daily for 5 mo.

Case 3: MP was switched to pan-enteral nutrition therapy. The patient was administered FLCZ 400 mg once daily for 1 mo.

OUTCOME AND FOLLOW-UP

Case 1: A chest CT upon completion of anti-fungal therapy showed reduced size of the pulmonary nodule in the left upper lobe.

Case 2: During anti-fungal therapy, the patient was started on thalidomide for CD treatment and reported no apparent abdominal pain. One month after anti-fungal therapy, he achieved symptom resolution, and a repeat chest CT scan showed lesion absorption (Figure 2B).

Case 3: IFX was initiated 7 years later because of the progression of CD. Regular follow-ups have revealed no recurrence so far.

The clinical courses of the three cases are summarized in Figure 4.

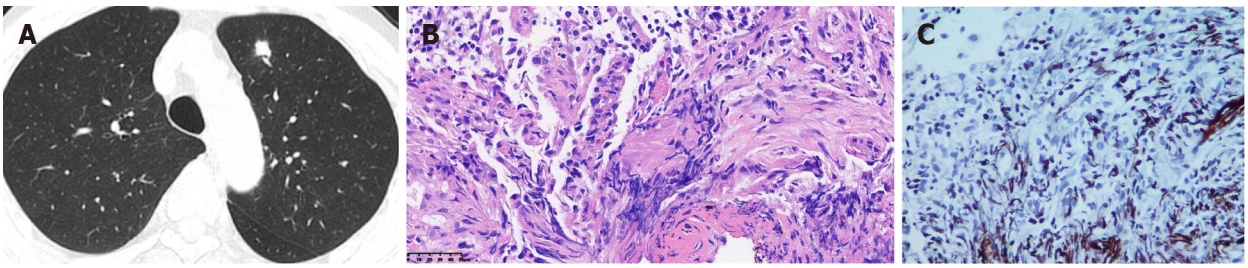
DISCUSSION

Cryptococcus infection is an opportunistic fungal infection caused by *Cryptococcus neoformans* or *Cryptococcus gattii*. These pathogens are ubiquitously distributed, with the respiratory tract being the primary portal of entry. Cryptococcosis often occurs in immunocompromised individuals, such as human

Table 1 Laboratory examination

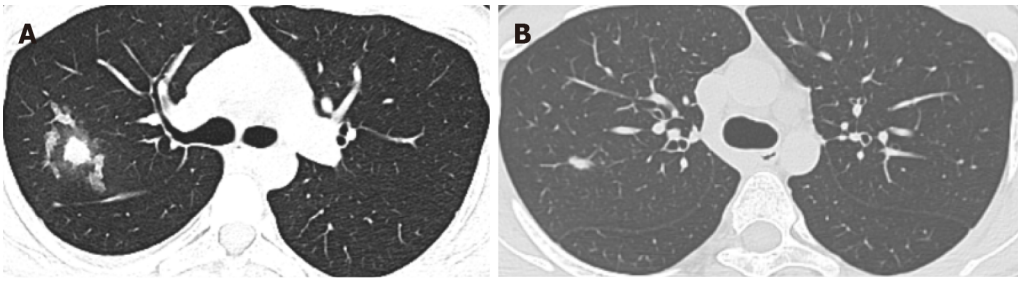
Case	1	2	3
WBC count (× 10^9/L)	10.0	9.4	2.5
Neutrophil count (× 10^9/L)	8.13	8.0	1.6
Platelet count (× 10^9/L)	305	282	220
Hemoglobin (g/L)	120	139	97
Albumin (g/L)	34.3	42.8	12.0
CRP (mg/L)	23.3	17.4	34.9
ESR (mm/hr)	19	16	14
Tumor markers	Negative	Negative	Negative
T-SPOT.TB	Negative	Negative	Weak positive
EBV IgM	Negative	Negative	-
Cytomegalovirus IgM	Negative	Negative	Negative
HIV	Negative	Negative	Negative
TPPA test	Negative	Negative	Negative

WBC: White blood cell; CRP: C reactive protein; ESR: Erythrocyte sedimentation rate; T-SPOT.TB: T-cell spot of tuberculosis test; EBV: Epstein-Barr virus; IgM: Immunoglobulin M; HIV: Human immunodeficiency virus; TPPA: Treponema pallidum particle agglutination.



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Figure 1 Imaging and pathologic findings of case 1. A: Chest computed tomography scan showing multiple nodules in bilateral lobes and a large one in the left upper lobe; B: Hematoxylin-eosin staining showing focal granuloma formation in alveolar tissues; C: Periodic acid-silver methenamine staining showing suspicious positive organisms.



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Figure 2 Imaging findings of case 2. A: Chest computed tomography (CT) scan before fluconazole treatment showing patchy lesions in the right upper lobe and right interlobar fissure and multiple nodules in bilateral lobes; B: Chest CT scan showing lesion absorption 6 mo after initiation of anti-fungal therapy.

immunodeficiency virus-infected patients, organ transplant recipients, and those with indications for immunosuppressants[3]. However, it has also been reported in immunocompetent patients[4].

A total of 21 studies reporting CD cases complicated by Cryptococcus infection were identified (Table 2)[4-24]. Among them, one was a retrospective analysis[21] (197 males and 69 females) and another reported relapse of a previously reported case[13]. The remaining 19 patients comprised 13 males and 6 females; 9 of the cases were disseminated cryptococcosis[4,5,8,12,15-19]. Infection sites

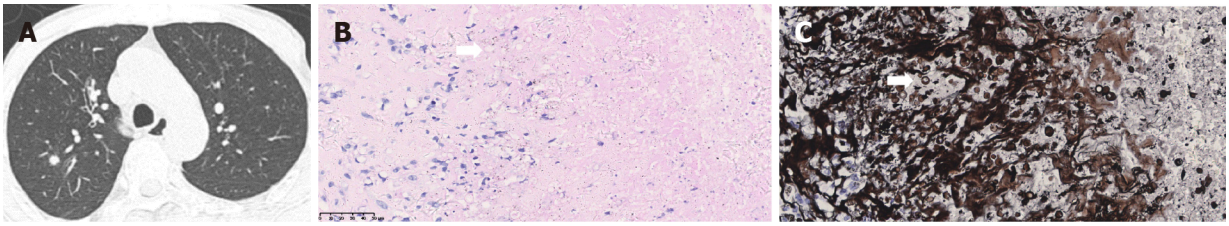
Table 2 Clinical characteristics of cryptococcus infection in patients with Crohn's disease

Ref.	Age/sex	Comorbidity	CD medication	Symptom	Infection site	Treatment	Outcome
Lerner *et al*[5], 1988	65/M	Ankylosing spondylitis	Prednisone	Calf ulcer	Lungs, skin	AmB, 5FC	Died
Hrnicek *et al*[6], 2003	51/M	None	IFX, prednisone, MTX, ciprofloxacin	Cough, fatigue, headache, fever, chills	Lungs	Surgery, FLCZ	Recovered
Rehman *et al*[7], 2008	61/M	None	IFX, prednisone, budesonide, AZA	None	Lungs	AmB, 5FC → FLCZ	Recovered
Osawa *et al*, 2010 [8]	53/M	Silicosis	IFX, prednisone, AZA	Abdominal pain, diarrhea, fever, night sweats, headache	Lungs, GI, CNS	AmB → LAmB, 5FC → FLCZ	Recovered
Sciaudone *et al* [4], 2011	26/F	None	None	Diarrhea, abdominal pain, weight-loss, headache, cough	Lungs, GI	Surgery, LAmB → FLCZ	Recovered
Hirai *et al*[9], 2011	39/M	None	IFX	None	Lungs	Surgery	Recovered
Takazono *et al* [10], 2012	35/M	None	IFX, prednisolone, mesalazine	Fever	Lungs	FLCZ	Recovered
Fraison *et al*[11], 2013	54/M	Ankylosing spondylitis	ADM, AZA	Fever, anorexia, cough, chest pain, dyspnea, arthralgia, myalgia	Lungs	LAmB, 5FC → FLCZ	Recovered
Wysocki *et al* [12], 2015	46/M	None	IFX, AZA →ADM →CZP	Abdominal and back pain, fever, headache	Omentum, CNS	LAmB, 5FC	Recovered
Takazono *et al* [13], 2016	35/M	None	IFX, prednisolone	Fever, fatigue, night sweats	Lungs	FLCZ → ITCZ	Recovered
Zhou *et al*[14], 2016	66/M	Cryptogenic organizing pneumonia	Prednisone	Dyspnea, cough	Lungs	FLCZ	Recovered
Saad *et al*[15], 2016	71/M	None	IFX, AZA	Meningitis	CNS	-	-
Vasant *et al*[16], 2016	74/F	Posterior reversible encephalopathy syndrome	IFX, prednisolone	Headache, confusion, fever, rigor, body ache	CNS	LAmB, 5FC → VRCZ	Died
Lee *et al*[17], 2017	70/F	*Klebsiella pneumoniae* and *Pneumocystis jiroveci* infections	IFX	Pyrexia	CNS	AmB → VRCZ	Died
Chavapradit *et al* [18], 2018	64/F	None	Prednisolone, AZA, mesalazine	Abdominal pain, diarrhea	Lungs, GI	AmB → FLCZ	Recovered
Maleb *et al*[19], 2019	45/M	None	Steroid, AZA	Shock, abundance of ascites, fever	Pleural fluid, ascites	FLCZ	Died
Santo *et al*[20], 2019	23/M	Tuberculosis	IFX, AZA	Fever, headache	Lungs	FLCZ	Recovered
Yeh *et al*[22], 2021	57/F	Systemic lupus erythematosus	ADM, prednisolone, mesalazine	Pneumonia	Lungs	AmB, 5FC	Recovered
Hussein *et al* [23], 2021	54/M	None	IFX, prednisolone, MTX	Fever, fatigue, cough	Lungs	FLCZ	Recovered
Yeoh *et al*[24], 2022	52/F	Autoimmune hepatitis	Prednisolone	Dyspnea, chest pain, cough, lethargy	Lungs	Surgery	Recovered

M: Male; F: Female; IFX: Infliximab; MTX: Methotrexate; AZA: Azathioprine; ADM: Adalimumab; CZP: Certolizumab pegol; GI: Gastrointestinal tract; CNS: Central nervous system; AmB: Aamphotericin B; LAmB: Liposomal amphotericin B; 5FC: 5-fluorocytosine; FLCZ: Fluconazole; ITCZ: Itraconazole; VRCZ: Voriconazole; -: Not reported.

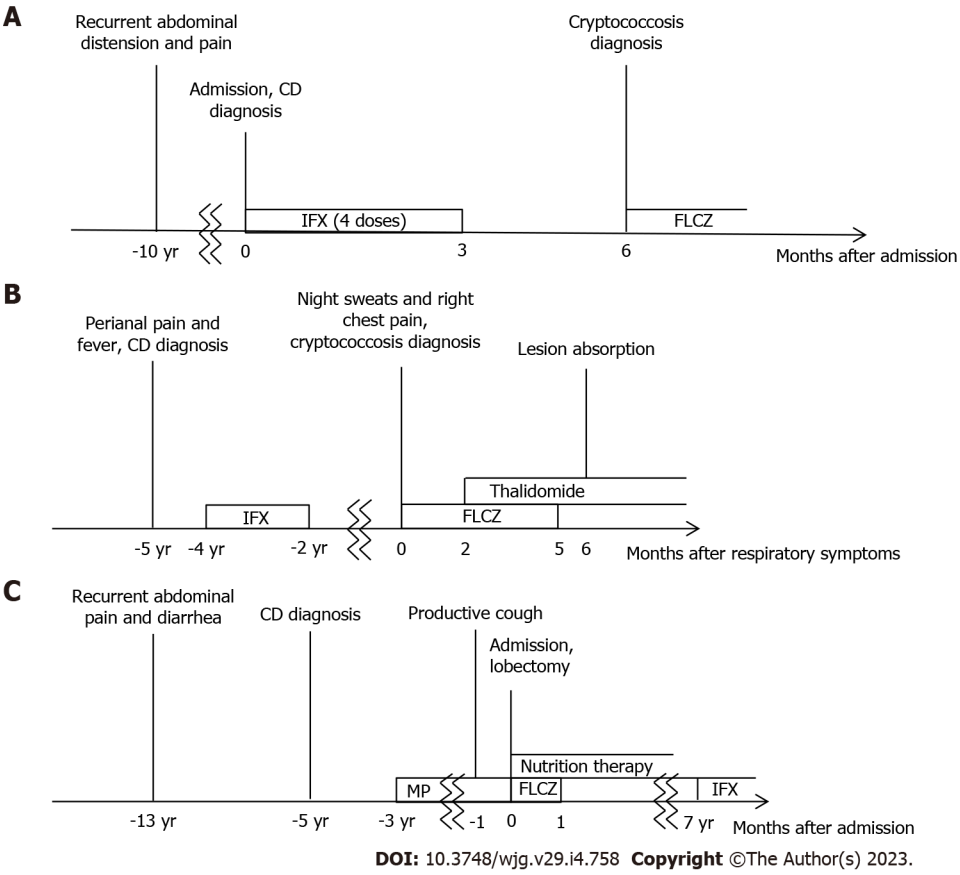
included lungs (15 cases), central nervous system (CNS; 5 cases), gastrointestinal tract (GI; 3 cases), skin (1 case), omentum (1 case), and pleural fluid and ascites (1 case).

Infected patients had been treated with different combinations of CD drugs, including anti-tumor necrosis factor α (TNF-α) agents, such as IFX, adalimumab and certolizumab pegol, corticosteroids, and other immunosuppressants. Symptoms ranged from asymptomatic lung granulomas to severe



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Figure 3 Imaging and pathologic findings of case 3. A: Chest computed tomography scan showing nodules in the posterior segment of right upper lobe; B and C: Periodic acid-Schiff and periodic acid-silver methenamine staining showing positive organisms (white arrows).



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Figure 4 Clinical courses of the three cases. A: Case 1; B: Case 2; C: Case 3. CD: Crohn's disease; IFX: Infliximab; FLCZ: Fluconazole; MP: Methylprednisolone.

pneumonia. Patients with disseminated cryptococcosis may also present with symptoms of other organ systems, such as colitis[8], meningitis[12], or skin ulcers[5]. Chest imaging typically revealed single or multiple pulmonary nodules. Diagnosis mainly relies on pathological examination, with accessory methods including fungal culture and the cryptococcal polysaccharide capsular antigen test. The most commonly used anti-fungal medications are amphotericin B (AmB), liposomal AmB (LAmB), 5-flucytosine (5FC), FLCZ, itraconazole, and voriconazole. Patients with mild symptoms generally recovered after treatment with triazole, while AmB and 5FC were intended for deep fungal infections; however, AmB and LAmB may cause serious adverse events[4,8]. Patients with disseminated cryptococcosis have a relatively poor prognosis, particularly when the CNS is involved.

Anti-TNF-α antibodies have been widely administered among CD patients with high efficacy, but concerns remain regarding their immunogenicity, skin toxicity, and increased risk of infection, such as the possible activation of latent TB. Therefore, current guidelines suggest that TB screening should be conducted before medication infusion[25]. TNF-α also plays a role in clearing pulmonary Cryptococcus infections by inducing interleukin-12 and interferon γ to promote a T1-cell-mediated immune response [26]. Anti-TNF-α agents, among other immunomodulators, may interfere with this process and increase the risk of *Cryptococcus* infection.

In this paper, we report three male patients diagnosed with CD, aged 20–65 years, who developed pulmonary cryptococcosis after being treated with anti-TNF-α agents or corticosteroids. The patients presented with asymptomatic or mild pneumonia with pulmonary nodules. One patient (case 2) showed elevated CA-125 level, which might be due to intestinal inflammation. G test and GM test were both negative, possibly because of low sensitivity of the tests for *Cryptococcus*. One patient (case 3) underwent surgery because of difficulty in biopsy. Diagnosis depended on postoperative pathological examinations since BALF might fail to detect *Cryptococcus*. All patients achieved symptom resolution after FLCZ treatment, consistent with other studies[6,10,23].

CD complicated with pulmonary cryptococcosis is relatively rare, but its incidence has increased in recent years with the use of various immunomodulators, particularly biologics, in CD management. Diagnosis is challenging because clinical symptoms and chest imaging both lack specificity, and GI manifestations can resemble CD progression, leading to misdiagnosis. Therefore, high clinical vigilance is required for early identification of infection, discontinuation of immunomodulators, and standardized treatment.

CONCLUSION

Our three case reports and literature review suggest that patients with timely diagnosis generally have a good prognosis. However, comorbidities, advanced age, and dissemination phenotype should raise clinical alerts. Metagenomic sequencing can be an alternative approach for pathogen diagnosis when biopsy is unfeasible.

FOOTNOTES

Author contributions: Fang YF and Cao Q contributed to conceptualization; Fang YF and Cao XH contributed to data collection and manuscript drafting; Fang YF, Cao XH and Yao LY contributed to manuscript revision; All authors have approved the final manuscript.

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ORCID number: Yan-Fei Fang 0000-0001-9292-7206; Qian Cao 0000-0001-7938-7532.

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ABOUT COVER

Editorial Board Member of *World Journal of Gastroenterology*, Jian-Gao Fan, PhD, Professor and Director, Center for Fatty Liver Disease, Department of Gastroenterology, Xinhua Hospital, Shanghai Jiaotong University School of Medicine; Shanghai Key Laboratory of Children's Digestion and Nutrition, Shanghai 200092, China. fattyliver2004@126.com

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EDITORIAL

Importance of human leukocyte antigen antibodies and leukocyte antigen/killer-cell immunoglobulin-like receptor genes in liver transplantation

Manuel Muro, Isabel Legaz

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Manuel Muro, Immunology Service, University Clinical Hospital Virgen de la Arrixaca-Biomedical Research Institute of Murcia (IMIB), Murcia 30120, Spain

Isabel Legaz, Department of Legal and Forensic Medicine, Biomedical Research Institute (IMIB), Regional Campus of International Excellence "Campus Mare Nostrum," Faculty of Medicine, University of Murcia, Murcia 30120, Spain

Corresponding author: Manuel Muro, PhD, Senior Researcher, Immunology Service, University Clinical Hospital Virgen de la Arrixaca-Biomedical Research Institute of Murcia (IMIB), El Palmar, Murcia 30120, Spain. manuel.muro@carm.es

Abstract

Many mechanisms have been proposed to explain the hypothetical state of hepatic tolerance, which is described by eventual imbalances or deregulation in the balance of cytokines, mediators, effectors, and regulatory cells in the complex milieu of the liver. In this section, we will comment on the importance of donor-specific anti-human leukocyte antigen (HLA) antibodies (DSA) as well as the compatibility and pairings of HLA and killer-cell immunoglobulin-like receptor (KIR) genotypes in the evolution of liver transplantation. Thus, HLA compatibility, viral infections, and HLA-C/KIR combinations have all been linked to liver transplant rejection and survival. There have been reports of increased risk of acute and chronic rejection with ductopenia, faster graft fibrosis, biliary problems, poorer survival, and even *de novo* autoimmune hepatitis when DSAs are present in the recipient. Higher mean fluorescence intensity (MFI) values of the DSAs and smaller graft size were associated with poorer patient outcomes, implying that high-risk patients with preformed DSAs should be considered for selecting the graft placed and desensitization methods, according to the investigators. Similarly, in a combined kidney-liver transplant, a pretransplant with a visible expression of several DSAs revealed that these antibodies were resistant to treatment. The renal graft was lost owing to antibody-mediated rejection (AMR). The HLA antigens expressed by the transplanted liver graft influenced antibody elimination. Pathologists are increasingly diagnosing AMR in liver transplants, and desensitization therapy has even been employed in situations of AMR, particularly in patients with DSAs in kidney-hepatic transplants and high-class II MFI due to Luminex. In conclusion, after revealing the negative impacts of DSAs with high MFI, pretransplant virtual crossmatch techniques may be appropriate to

improve evolution; however, they may extend cold ischemia periods by requiring the donor to be typed.

Key Words: Acute rejection; Alloantibodies donor-specific antibodies-donor-specific anti-human leukocyte antigen antibodies; Chronic rejection; Human leukocyte antigen matching; Killer-cell immunoglobulin-like receptor matching; Liver transplant

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Core Tip: This editorial aimed to raise realities, doubts, and ambiguities in the fundamental role of alloantibodies and the compatibility and association of the proteins encoded by the human leukocyte antigen and killer-cell immunoglobulin-like receptor genes in liver transplantation.

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INTRODUCTION

In the vast majority of transplants performed today, there is a clear demonstration of the role played by the best human leukocyte antigen (HLA) compatibility and the absence of donor-specific anti-HLA antibodies (DSA) in its positive evolution, and it is an increasingly important role. The significance of the role of compatibility and killer-cell immunoglobulin-like receptor (KIR) genotypes (especially in hematopoietic stem cell transplantation) has not been demonstrated in the case of liver transplantation. This suggests that the classic concept of the liver is different and may be an "immunologically privileged" organ. Transplant (even if there is a positive pretransplant crossmatch and DSAs are known) without accounting for donor and recipient typing can lead to antibody-mediated rejection (AMR)[1]. However, there are articles where this has been re-evaluated, and new essential effects of antibodies and compatibility in acute rejection, chronic rejection (CR), fibrosis, and liver transplant survival appear.

The hypothetical state of tolerance of the liver has been explained by many causes (profusely explained in an article of its own), and is explained by eventual imbalances or deregulations in the balance of cytokines, mediator, effectors, and regulatory cells in the complex microenvironment of the liver, including increased or decreased expression of costimulatory or soluble molecules, specific genetic profiles, or even a protective role of Kupffer cells[2-9].

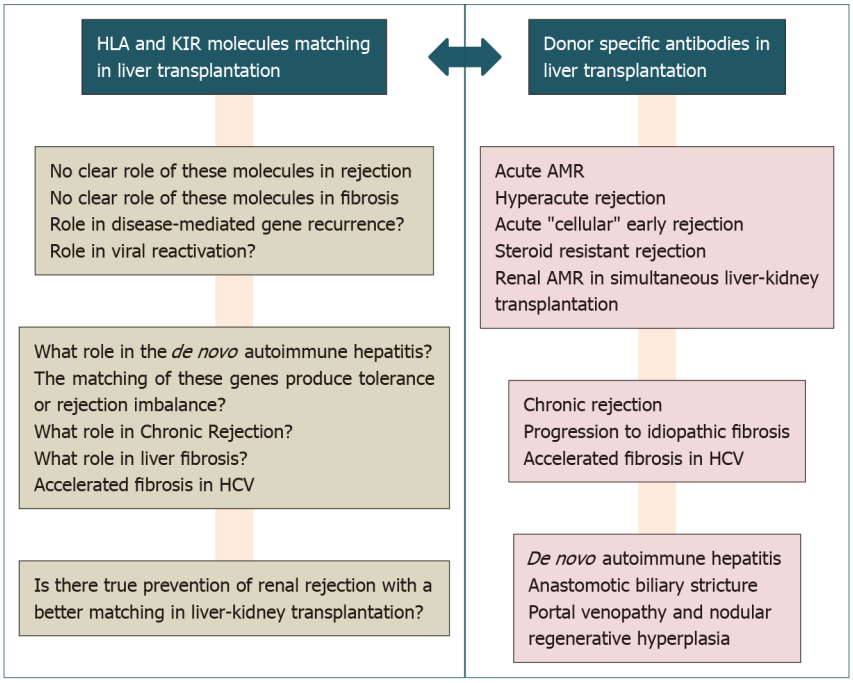
Here we focused on commenting on the role of DSA antibodies and the compatibility and pairings of HLA and KIR genotypes with the evolution of liver transplantation. Thus, HLA compatibility, viral infections, and HLA-C/KIR combinations have been classically related to liver transplant rejection and survival[10-13].

HLA ANTIBODIES AND *HLA*/*KIR* GENES IN LIVER TRANSPLANT IMMUNOLOGY

Regarding the existence of DSA antibodies present in the recipient, there are reports of increased risk of acute rejection and CR with ductopenia, accelerated graft fibrosis, biliary complications, worse survival, and even *de novo* autoimmune hepatitis[2,14]. However, some series and research groups reported different results and disparate causes (Figure 1). However, the literature on the role of DSAs and AMR is limited to clinical cases and small series[15].

Regarding preformed antibodies in the recipient before implantation, there is literature that reveals that patients with preformed DSA presented a worse graft evolution in living donor transplantation [16]. Higher mean fluorescence intensity (MFI) values of the DSAs and small graft size were associated with worse patient outcomes, suggesting to the authors that high-risk patients with preformed DSAs should be considered for selecting the graft implanted and desensitization protocols. Likewise, a pretransplant with the tangible expression of multiple DSAs[17] in a combined kidney-liver transplant showed that these antibodies were refractory to treatment, and the renal graft was lost due to AMR. The elimination of the antibodies depended on the HLA antigens expressed by the implanted liver graft.

In this sense, pathologists diagnose AMR in liver transplants with increasing frequency, and desensitization therapy has even been used in AMR cases, especially in patients with DSAs in kidney-hepatic



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Figure 1 Potential associations of donor-specific anti-human leukocyte antigen antibodies and human leukocyte antigen and/or matching with the evolution of liver transplantation. AMR: Antibody-mediated rejection; HCV: Hepatitis C virus; HLA: Human leukocyte antigen; KIR: Killer-cell immunoglobulin-like receptor.

transplants with high-class II MFI due to Luminex[18-20].

Although it is daring to assert categorically that the presence of DSAs contraindicates transplantation due to the same scientific literature, which is disparate between series, authors, and transplant centers, it is not well-defined over time (studies of positive, negative, and neutral papers) and the best methods of antibody diagnosis, evaluation of biopsies, and anti-rejection treatments[15]. In this way, regular DSA-post-transplant monitoring cannot as yet be recommended in routine practice but may be helpful in selected cases.

In the case of combined kidney transplants, there is also controversy and disparity between studies and groups. Thus, pretransplant DSAs increase the risk of AMR in the kidney and liver and worsen survival[12], with no data on the case of heart and lung combined with the liver. It has also been observed that the pretransplantation presence of anti-HLA class II antibodies and especially with positive complement fixation C1q or C3d have a risk of early AMR and a worse evolution of the transplant due to association of the graft with deposits of C4d in sinusoidal endothelial cells, increased fibrosis, CR, cirrhosis, and centrilobular fibrosis[2,13,16].

Regarding the development of *de novo* DSA (dnDSA), it has been estimated that immunosuppression may also play a role in the development of dnDSA. Thus, the coefficient of tacrolimus variation and mean tacrolimus levels have been reported to be associated with no dnDSA generation[21].

Other authors found that patients with an immunosuppressive regimen without withdrawal calcineurin inhibitors (mTOR inhibitors and/or maintenance with mycophenolic acid) have a higher prevalence of developing dnDSA post-transplant than patients with a standard regimen[22]. However, dnDSAs with calcineurin-free immunosuppression were associated with normal graft histology. The use of rituximab induction among DSA recipients has also been considered[23]. A dose of rituximab > 300 mg/m^2 was well tolerated and achieved a lower incidence of AMR.

In addition, everolimus combined with tacrolimus was associated with negative HLA and DSA antibody status[24]. Viral etiology of liver disease, hepatocellular carcinoma, and higher degrees of graft steatosis were associated with a lower rate of HLA antibodies. The impact of HLA and DSA antibodies was associated with higher levels of transaminases and bilirubin. In addition, a significant association was detected between higher degrees of inflammation and the presence of HLA and DSA antibodies. Thus, DSA would be associated with histological and biochemical inflammation of the graft after liver transplantation, while fibrosis seems unaffected.

There are also cases in the literature of living donor liver transplants who developed acute AMR after desensitization to perform DSA and were successfully treated with bortezomib and everolimus therapy [25]. In this regard, in sensitized combined liver-kidney transplant recipients, the “delayed” kidney transplant approach was associated with a significant reduction in total and class I DSAs after liver transplantation before kidney transplantation[26], allowing therapeutic interventions such as plasmapheresis, providing optimal results similar to those of crossmatched recipients.

Finally, regarding single or triple-therapy monotherapy, it has been reported that the development of class II DSA occurs more often with immunosuppressive monotherapy and may ultimately result in chronic rejection and graft fibrosis[27].

On the other hand, Shin *et al*[21] found that patients without T-cell rejection in pediatric liver transplantation were more likely to have dnDSAs for HLA-DQ7 and less likely to have these DSAs for HLA-DQ2. Therefore, they deduced that a load of mismatched epitopes predicted the non-generation of these DSAs. At the same time, the specificity of *de novo* DSAs could determine alloimmunity.

Also, references for the location and the importance of the correct detection of these DSAs would corroborate that the existence of intragraft DSA and intragraft union reaction of C3d (using a fluorescent analysis technique of capture of immunocomplexes) harms the outcome of the transplant, unlike DSA present in serum, with no impact[28].

Finally, it has been reported that the incidence of DSA after liver transplantation is higher in children than in adults, that DSAs directed against HLA class II molecules, mainly DQ, occur more often, and that the presence of such anti-class II DSA (DQ/DR), especially of the complement-binding IgG3 subclass, may be associated with endothelial injury, T-cell-mediated rejection (TCMR), inflammation, and fibrosis[29-31].

Regarding the positive, negative, or neutral role of the compatibility of the *HLA* and/or *KIR* genes, it is a subject of almost as much debate as the subject of antibodies. Historical studies have commented on any of the possibilities[10-12,32], and at the moment, there is no consistency in all the studies reviewed in this editorial. Regarding the role of HLA incompatibility and the evolution of the liver allograft, it is not separate from promoting the development of DSAs, with the logical criterion that the more incompatibilities, the more possibilities exist to develop antibodies DSAs *de novo*. Thus, the new molecular HLA incompatibility (MM) improves the prediction of the evolution of the transplant. Thus, in a study by Ono *et al*[33] on liver transplantation from a living donor, the risk of TCMR and the development of dnDSA were evaluated using eplets. MM in HLA-DQB1 eplets was associated with TCMR. The predicted indirectly recognizable HLA epitopes II (PIRCHE-II) score for the *HLA-DQB1* gene was also significantly higher in patients with TCMR. Moreover, DQB1-EpMMs ≥ 9 and DQB1-predicted indirectly recognizable HLA epitopes II score ≥ 3 were predictors of dnDSA formation. Thus, MM analysis may be applied toward tailored immunosuppression based on individual risks.

In this sense, a very recent article[34] on living donor transplants found that the more HLA incompatibilities there are, the worse the patient's survival was (for A + B + DR, A + B + C, DR + DQ, and A + B + C + DR + DQ). For HLA-B + DR mismatches, the risk of a TCMR was more pronounced in adults but not in children. It has also been reported in 1042 liver transplants and 9.38 years of follow-up that HLA-A mismatch was strongly associated with graft failure and mortality, especially with two mismatches [35].

However, other groups commented that incompatibility was not associated with acute rejection, early allograft dysfunction, or survival in living donor liver transplants[36]. The impact of HLA-A and HLA-DR incompatibility on cytomegalovirus reactivation and sepsis were significant but with very low significance and were not conclusive.

There is very little published and consistent literature on KIR compatibility, particularly in liver transplantation[10,16,32,37-41]. From more recent authorship, we know that the incidence of acute rejection does not correlate with HLA compatibility nor with KIR alleles or genotypes of the recipient, but the frequency of C2+ donors did increase in the rejection group and was more frequent when the recipient expressed KIR2DS4[39].

In another study, grafts from donors without *HLA-C2* alleles produced more rejection than in recipients from donors with at least one *HLA-C2* allele[42], consistent with a previous study of ours[32], which showed that *HLA-C2* homozygotes receiving HLA-C1/C2 grafts had a higher risk of rejection than *HLA-C1* homozygotes. Other groups, however, did not find this association in their series[38], so the issue is still under open debate.

CONCLUSION

In conclusion, after demonstrating the adverse effects of DSAs with high MFI, perhaps pretransplant virtual cross match protocols could be appropriate to improve evolution, although they could increase cold ischemia times by having to type the donor. Although today, there is no particular problem as the times of typing results have been shortened, which also allows the optimization of compatibility and *HLA* and *KIR* genotypes[15,43].

In our modest opinion, monitoring of dnDSAs should also be universally adopted in all transplant centers to avoid possible post-transplant complications as much as possible. More extensive cohort studies, including the MFI intensity of each DSA in the donor, the role of the different HLA and KIR compatibility, and particular combinations between donor and recipient, are needed to clarify their actual role in the post-transplant period.

FOOTNOTES

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Country/Territory of origin: Spain

ORCID number: Manuel Muro 0000-0001-9987-0994; Isabel Legaz 0000-0002-1140-4313.

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OPINION REVIEW

Management of gastro-esophageal reflux disease: Practice-oriented answers to clinical questions

Leonardo Frazzoni, Lorenzo Fuccio, Rocco Maurizio Zagari

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Leonardo Frazzoni, Lorenzo Fuccio, Rocco Maurizio Zagari, Department of Digestive Diseases, IRCCS Azienda Ospedaliero-Universitaria di Bologna Policlinico S Orsola, Bologna 40138, Italy

Lorenzo Fuccio, Rocco Maurizio Zagari, Department of Medical and Surgical Sciences, University of Bologna, Bologna 40138, Italy

Corresponding author: Leonardo Frazzoni, MD, Academic Fellow, Medical Assistant, Department of Digestive Diseases, IRCCS Azienda Ospedaliero-Universitaria di Bologna Policlinico S Orsola, Via Albertoni 15, Bologna 40138, Italy. leonardo.frazzoni@gmail.com

Abstract

Gastro-esophageal reflux disease (GERD) is a condition which is frequently faced by primary care physicians and gastroenterologists. Improving management of GERD is crucial to maximise both patient care and resource utilization. In fact, the management of patients with GERD is complex and poses several questions to the clinician who faces them in clinical practice. For instance, many aspects should be considered, including the appropriateness of indication to endoscopy, the quality of the endoscopic examination, the use and interpretation of ambulatory reflux testing, and the choice and management of anti-reflux treatments, *i.e.*, proton-pump inhibitors and surgery. Aim of the present review was to provide a comprehensive update on the clinical management of patients with GERD, through a literature review on the diagnosis and management of patients with GER symptoms. In details, we provide practice-oriented concise answers to clinical questions, with the aim of optimising patient management and healthcare resource use.

Key Words: Gastro-esophageal reflux disease; Diagnosis; Management; Proton-pump inhibitor

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Core Tip: Gastro-esophageal reflux disease (GERD) still poses several clinical issues to be faced, from clinical and instrumental diagnosis to medical and surgical therapy. In this review we provide the most updated evidence on the management of GERD. Practice-oriented questions on GERD are answered through a concise review of current literature. The aim is to provide clinicians a practical tool to guide them through the management of patients with GERD.

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INTRODUCTION

Gastro-esophageal reflux disease (GERD) is a complex but common condition[1] that poses several issues to the clinicians. Prompt endoscopy should be reserved only to patients with symptoms of GERD and alarm features or multiple risk factors for Barrett's esophagus. Grade A esophagitis is not sufficient to diagnose GERD, and only patients with grade C and D esophagitis should undergo endoscopic follow-up after proton-pump inhibitors (PPIs). Evidence of posterior laryngitis is not reliable for diagnosing GERD. Reliable selection of patients with PPI-refractory GERD who can benefit from anti-reflux surgery is a critical issue and relies on careful evaluation including impedance-pH monitoring. Prokinetics may be used in patients with concomitant dyspeptic symptoms, whereas potassium-competitive acid blockers (P-CABs) may be an option for erosive esophagitis.

QUESTION 1: SHOULD I PERFORM ENDOSCOPY IN ALL PATIENTS WITH GERD SYMPTOMS?

Answer: According to more recent international guidelines, a clinical response to an empiric 8-wk once-daily PPI therapy is diagnostic for GERD in patients with heartburn or acid regurgitation[2,3]. This pragmatic approach has a sensitivity of 78% and specificity of about 54%[4], which means to avoid unnecessary endoscopy in more than half of patients with symptoms of GERD. In fact, it should be emphasised that most patients with confirmed GERD do not present endoscopic findings of erosive esophagitis[2]. On the other hand, prompt endoscopy is recommended for patients with GERD symptoms and dysphagia or other alarm features (*e.g.*, weight loss, vomiting, or signs of gastrointestinal bleeding). Endoscopy is also recommended in all patients with GERD symptoms and at least 2 of the following risks factors for Barrett's esophagus: Age ≥ 50 years, male gender, Caucasian ethnicity, obesity, family history for Barrett's esophagus or esophageal adenocarcinoma, and smoking[2,5]. Indeed, the prevalence of Barrett's esophagus among patients with GERD symptoms is only about 5%-7%[6,7], therefore endoscopy should be reserved to patients with multiple risk factors for this condition.

QUESTION 2: IS EROSIVE ESOPHAGITIS SPECIFIC FOR DIAGNOSIS OF GERD?

Answer: Traditionally, endoscopic erosive esophagitis is considered specific for the diagnosis of GERD. The Los Angeles (LA) classification is currently the most used one for grading erosive esophagitis and considers 4 degrees: Grade A and B, non-confluent erosions (*i.e.*, mucosal breaks) of longitudinal extension ≤ 5 mm or > 5mm, respectively; grade C and D, confluent erosions between multiple folds affecting < 75% or ≥ 75% of the circumference, respectively[8]. According to recent international guidelines, the presence of grade A erosive esophagitis is not sufficient to diagnose GERD, as it can be present in 5%-8% of healthy subjects who do not experience symptoms of GER nor present complications such as Barrett's esophagus, and can be linked to other factors such as drugs or infections[2,3]. Grade B esophagitis can be considered diagnostic of GERD in the presence of typical symptoms of GERD that respond to PPI therapy, while grade C and D esophagitis are always diagnostic for GERD [2]. Nevertheless, it should be noted that erosive esophagitis is mostly healed by PPI therapy, therefore PPIs should be stopped at least 2 wk before endoscopy[2].

QUESTION 3: WHEN TO PERFORM ESOPHAGEAL BIOPSIES IN PATIENTS WITH GERD SYMPTOMS?

Answer: Esophageal biopsies are currently not considered in patients with GERD symptoms as they are of little value for the diagnosis of GERD. Histopathological findings that are variably associated with GERD, including dilation of the intercellular spaces and inflammatory intraepithelial cells and necrosis, have been described in the literature[9], but are flawed by a suboptimal specificity[10]. In fact, esophageal biopsies should be performed to diagnose eosinophilic esophagitis. This condition might coexist when patients refer also dysphagia and food bolus impaction in the esophagus. In this case, at least 6 biopsies should be performed in multiple esophageal sites[11]. Since PPIs can mask endoscopic and histological features of eosinophilic esophagitis, PPI therapy should be stopped at least 2 wk before endoscopy.

QUESTION 4: SHOULD HIATAL HERNIA ALWAYS BE IDENTIFIED AND MEASURED?

Answer: The systematic identification and measurement of hiatal hernia is important for several reasons: (1) Hiatal hernia is a predisposing factor for GERD; (2) If present, it should be corrected during laparoscopic fundoplication when technically feasible; and (3) Measurement of hiatal hernia presupposes the correct identification of landmarks, *i.e.*, diaphragmatic hiatus and esophago-gastric and squamocolumnar junctions, in turn necessary for the correct diagnosis of Barrett's esophagus. Therefore, although some evidence suggests that endoscopy is not the test of choice for measuring hiatal hernia [12], it is important to standardise this procedure to maximise its accuracy and reliability. First, endoscopy must be performed under sedation to avoid retching that could temporarily displace the gastric fundus. Second, the measurement must be carried out between the diaphragmatic hiatus and the top of the gastric folds (*i.e.*, esophagus-gastric junction). Last, excessive insufflation should be avoided, and the measurement should be always carried out during the same phase of the examination, *i.e.*, during extubation in order to minimise the effect of gastric prolapse following intubation.

QUESTION 5: SHOULD PATIENTS WITH EROSIVE ESOPHAGITIS UNDERGO REPEAT ENDOSCOPY AFTER TREATMENT?

Answer: The rationale for repeating endoscopy after treatment in patients with erosive esophagitis is mainly linked to the possibility that inflammation could obscure the visibility of an underlying Barrett's esophagus. Secondly, in those with more severe erosive esophagitis (LA grade C or D) it is advisable to check for the healing of the lesions and possible occurrence of complications (*e.g.*, peptic stricture) after adequate therapy with PPIs. Barrett's esophagus at repeat endoscopy after PPI treatment for erosive esophagitis has been reported in up to 12% of cases[13]. However, Barrett's esophagus is mostly obscured by LA grade C and D esophagitis, with a lower incidence reported in grades A and B[13]. Therefore, guidelines currently recommend repeating endoscopy after an 8-wk course of PPI therapy only in patients with LA grade C and D erosive esophagitis[14].

QUESTION 6: IS AN INSTRUMENTAL FINDING OF LARYNGITIS A SPECIFIC SIGN OF GERD?

Answer: The extra-esophageal manifestations of GERD are various and their association with GERD cannot always be unequivocally proven. Some findings at laryngoscopy, such as erythema and oedema of the vocal cords or larynx, may be related to GERD, but the specificity of these signs for the diagnosis of GERD is as low as 40%[15]. These findings may be attributable to other conditions, such as post-nasal drip syndrome or exposure to allergens and other environmental irritants[15]. Furthermore, the response to PPI therapy in these patients is unreliable due to the large placebo effect. Therefore, the presence of laryngeal symptoms (*e.g.*, cough, hoarseness), even when associated with an instrumental finding of laryngeal inflammation, is not sufficient for the diagnosis of GERD, and patients should be referred for further diagnostic investigations to confirm this diagnosis, *e.g.*, endoscopy if not previously performed and/or impedance-pH monitoring[2].

QUESTION 7: IS PH MONITORING ALONE INFERIOR TO IMPEDANCE-PH MONITORING TO DIAGNOSE GERD?

Answer: Ambulatory reflux monitoring, including pH-monitoring and impedance-pH monitoring, is the method of choice to confirm or exclude the diagnosis of GERD[2,3]. Impedance detects the movement of fluids and gas inside the esophagus independently from their acidity, thus distinguishing weakly acid from acid refluxes and reliably documenting the total number of reflux events throughout the recording period. Two additional applications need to be briefly mentioned: The post-reflux swallow-induced peristaltic waves (PSPW) index is the ratio between reflux episodes timely followed by a swallow event, and all the reflux episodes; this measure assesses esophageal chemical clearance due to the esophago-salivary reflex and has been shown to be impaired in GERD[16,17]. The mean nocturnal basal impedance (MNBI) is the mean baseline impedance value in three 10-min periods from the most distal impedance channel during nighttime recumbent period; this measure assesses the integrity of esophageal mucosa and is reduced by the chronic inflammation due to GERD[16,17].

Recent evidence has shown that pH monitoring alone, using esophageal acid exposure time (AET) > 6% according to the Lyon consensus[3] confirms the diagnosis of GERD only in 45% of patients with PPI-responsive heartburn[17]. On the other hand, impedance-pH monitoring with the evaluation of total refluxes, MNBI and PSPW index increases the diagnostic yield of about 20%, especially allowing to better characterise patients with inconclusive AET between 4% and 6%[17]. Of note, impedance-pH monitoring can identify ongoing reflux in a much higher proportion of PPI-refractory patients than pH monitoring alone, when performed on-therapy[18]. Therefore, impedance-pH monitoring should be considered the test of choice to confirm or rule out a diagnosis of GERD.

QUESTION 8: HOW SHOULD I MANAGE PPI THERAPY BEFORE IMPEDANCE-PH MONITORING?

Answer: The choice of performing impedance-pH monitoring off-PPI or on-PPI depends on the clinical goal. Impedance-pH monitoring should be performed off-PPI to demonstrate that pathological gastro-esophageal reflux underlies symptoms in a patient with unproven GERD[3]: That is when, for instance, a patient with normal endoscopic findings complains of typical or extra-esophageal symptoms and requires continuous PPI for symptom control or asks for anti-reflux surgery. On the other hand, impedance-pH monitoring should be performed on-PPI to confirm or exclude that ongoing reflux is the cause of inadequate response to double-dosage PPI in a patient with documented GERD[3].

QUESTION 9: WHEN SHOULD I VERIFY PATIENT ADHERENCE TO PPI THERAPY?

Answer: Modality and timing of PPI intake are key factors in obtaining an adequate response. Proton pump inhibitors should be taken at least 30 min before the first meal, preferably in the morning before breakfast, and in case of a second dose in the evening before dinner. This allows to achieve the maximum suppression of gastric acid secretion by inhibiting proton pumps before these are activated by food[19]. However, there is evidence that a large proportion of patients with unresponsive GERD symptoms do not take PPIs 30 min before the first meal[20]. Additionally, two studies found that only about half of patients correctly adhered to PPI therapy prescriptions for more than 80% of the time and that increasing compliance was typically related to symptom improvement[19]. Indeed, patient adherence to PPIs should be always verified in case of PPI-refractory symptoms.

QUESTION 10: WHEN DO I REFER A PATIENT WITH PPI-REFRACTORY SYMPTOMS TO ANTI-REFLUX SURGERY?

Answer: Patients with symptoms suggestive of gastro-oesophageal reflux unresponsive to PPIs should first be investigated about compliance and adherence to therapy. In case of good compliance, they should be referred for off-PPI upper gastrointestinal endoscopy and impedance-pH monitoring to confirm GERD diagnosis. Indeed, PPI therapy is so effective for typical GERD symptoms when properly administered that true PPI-refractoriness should prompt to verify the actual correlation between symptoms and reflux. On the other hand, in case of proven GERD impedance-pH monitoring should be performed on double-dosage PPI therapy started from at least 8 wk, in order to reliably link PPI-refractory symptoms to ongoing reflux and exclude reflux-unrelated symptoms. Indeed, in a recent randomised controlled trial (RCT) evaluating 366 patients referred for PPI-refractory heartburn only 21% of cases showed a clear-cut impedance-pH correlation between heartburn and gastro-esophageal reflux[21]. This highlights the importance to refer for surgical fundoplication only patients with PPI-

refractory GERD confirmed by impedance-pH monitoring. Correct selection of patients is crucial to maximise the outcome of anti-reflux surgery, which can be as high as 90%[18].

QUESTION 11: WHICH IS THE ROLE FOR PROKINETICS IN PATIENTS WITH GERD?

Answer: Dyspeptic symptoms can present in nearly half of patients with GERD, and the probability of dyspepsia in individuals with weekly GER symptoms is nearly 7-fold higher than in subjects without GERD[22]. There is a pathophysiological basis for this association, as prolonged postprandial gastric distention and increased basal intragastric pressure may lead to an increased gastro-esophageal pressure gradient, favoring reflux episodes. Therefore, prokinetics such as metoclopramide and domperidone may be beneficial when added to PPI therapy in patients with concomitant dyspeptic symptoms. However, the caveat is that their use can be limited by side effects including drowsiness, agitation, irritability, depression, dystonic reactions, and tardive dyskinesia for metoclopramide, whereas QT monitoring seems prudential for domperidone due to small risk for ventricular arrhythmia and sudden cardiac death[2].

QUESTION 12: WHICH IS THE ROLE FOR P-CABS IN PATIENTS WITH GERD?

Answer: P-CABs competitively inhibit proton pumps and have been licensed in Japan for the treatment of GERD since 2015[19]. Differently from PPIs, vonoprazan can block both inactive and active proton pumps, resulting in a higher and longer-lasting suppression of gastric acid secretion[19]. Further, its elimination is independent from CYP2C19 metabolism, probably contributing to explain its greater effect[19]. A recent meta-analysis on 19 RCTs found that vonoprazan was superior to PPIs in healing erosive esophagitis, whereas there was no difference in the improvement of GERD symptoms[23]. However, evidence on refractory GERD is scarce, and more studies from Western countries are needed to expand knowledge on the effectiveness of this drug in the setting of erosive reflux disease.

CONCLUSION

GERD is one of the most frequent gastroenterological conditions, yielding a considerable amount of resource consumption in health services[1]. Although several guidelines have been published[2,3], the management of patients with GER symptoms is still controversial. Currently, for example, there is no gold standard for diagnosing GERD, as diagnosis relies on a combination of symptoms, response to PPI therapy, endoscopy, and ambulatory reflux monitoring. Recent evidence-based recommendations provide new insights regarding erosive esophagitis and the management of patients refractory to PPIs [2,3]. This review provides the answers to questions which were selected after collegial discussion between the authors, also taking into account the most debated issues with general practitioners and non-dedicated gastroenterologists, that may help physicians in the management of patients with GERD (see Table 1). The answers are based on the overview of current guidelines and recommendations and on recent evidence provided from systematic reviews and clinical trials.

Table 1 Practice-oriented answers to clinical questions on the management of gastro-esophageal reflux disease

No.	Question	Answer
1	Should I perform endoscopy in all patients with GERD symptoms?	Endoscopy should be reserved for patients with GERD symptoms and either alarm features or multiple risk factors for Barrett's esophagus
2	Is erosive esophagitis specific for diagnosis of GERD?	Only LA grade C and D esophagitis are always specific for GERD
3	When to perform esophageal biopsies in patients with GERD symptoms?	Esophageal biopsies should be performed only when eosinophilic esophagitis is suspected
4	Should hiatal hernia always be identified and measured?	Hiatal hernia should always be identified and measured
5	Should patients with erosive esophagitis undergo repeat endoscopy after treatment?	Only patients with LA grade C and D esophagitis should undergo repeat endoscopy after PPI therapy
6	Is an instrumental finding of laryngitis a specific sign of GERD?	Laryngoscopic findings of laryngitis are not specific signs of GERD
7	Is pH monitoring alone inferior to impedance-pH	Impedance-pH monitoring is the test of choice to confirm or rule out GERD

	monitoring to diagnose GERD?	
8	How should I manage PPI therapy before impedance-pH monitoring?	The choice of performing impedance-pH monitoring off-PPI or on-PPI depends on the clinical goal
9	When should I verify patient adherence to PPI therapy?	Adherence to PPI therapy should be always verified in case of PPI-refractory symptoms
10	When do I refer a patient with PPI-refractory symptoms to anti-reflux surgery?	Only patients with PPI-refractory GERD confirmed by impedance-pH monitoring should be referred to surgical fundoplication
11	Which is the role for prokinetics in patients with GERD?	Prokinetics may be used in patients with GERD and concomitant dyspeptic symptoms
12	Which is the role for P-CABs in patients with GERD?	P-CABs are promising antisecretory drugs, however more evidence is needed

GERD: Gastro-esophageal reflux disease; PPI: Proton-pump inhibitor; LA: Los Angeles; P-CABs: Potassium-competitive acid blockers.

FOOTNOTES

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Country/Territory of origin: Italy

ORCID number: Leonardo Frazzoni 0000-0001-7084-6157; Lorenzo Fuccio 0000-0001-8618-2447; Rocco Maurizio Zagari 0000-0001-9949-8619.

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REVIEW

Transcriptome analysis creates a new era of precision medicine for managing recurrent hepatocellular carcinoma

Chun-Cheng Chiang, Hsuan Yeh, Siew-Na Lim, Wey-Ran Lin

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Chun-Cheng Chiang, UPMC Hillman Cancer Center, University of Pittsburgh, Pittsburgh, PA 15232, United States

Hsuan Yeh, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15213, United States

Siew-Na Lim, Department of Neurology, Linkou Chang Gung Memorial Hospital, Taoyuan 333, Taiwan

Siew-Na Lim, Wey-Ran Lin, College of Medicine, Chang Gung University, Taoyuan 333, Taiwan

Wey-Ran Lin, Department of Gastroenterology and Hepatology, Linkou Chang Gung Memorial Hospital, Taoyuan 333, Taiwan

Corresponding author: Wey-Ran Lin, MD, PhD, AGAF, Professor, Department of Gastroenterology and Hepatology, Linkou Chang Gung Memorial Hospital, No. 5 Fu-Shin Street, Taoyuan 333, Taiwan. t12360@adm.cgmh.org.tw

Abstract

The high incidence of hepatocellular carcinoma (HCC) recurrence negatively impacts outcomes of patients treated with curative intent despite advances in surgical techniques and other locoregional liver-targeting therapies. Over the past few decades, the emergence of transcriptome analysis tools, including real-time quantitative reverse transcription PCR, microarrays, and RNA sequencing, has not only largely contributed to our knowledge about the pathogenesis of recurrent HCC but also led to the development of outcome prediction models based on differentially expressed gene signatures. In recent years, the single-cell RNA sequencing technique has revolutionized our ability to study the complicated crosstalk between cancer cells and the immune environment, which may benefit further investigations on the role of different immune cells in HCC recurrence and the identification of potential therapeutic targets. In the present article, we summarized the major findings yielded with these transcriptome methods within the framework of a causal model consisting of three domains: primary cancer cells; carcinogenic stimuli; and tumor microenvironment. We provided a comprehensive review of the insights that transcriptome analyses have provided into diagnostics, surveillance, and treatment of HCC recurrence.

Key Words: Recurrent hepatocellular carcinoma; Microarrays; RNA sequencing; Single-cell RNA sequencing; Precision medicine; Tumor heterogeneity; Tumor microenviron-

ment

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Core Tip: The high incidence of hepatocellular carcinoma (HCC) recurrence seriously threatens patient outcomes. This review detailed how various transcriptome profiling methods have contributed to our understanding of recurrent HCC with respect to the carcinogenicity of primary cancer cells, carcinogenic stimuli, and tumor microenvironments, which show great promise in improving the management of HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for 75%-85% of primary liver cancer caused by chronic liver injury[1]. The advance in surgical techniques and locoregional liver-directed therapies contributes to the prognosis of patients suffering from early HCC. However, high-relapse HCC remains a serious burden to patients treated with curative intent, as the annual recurrence rate of HCC following surgery is 50%-70% within 5 years[2-4]. Although recent progress in systemic treatments has led to the modification of treatment strategy for intermediate to advanced HCC[5], early detection of HCC recurrence can provide patients with more treatment options. It is therefore imperative to identify susceptible patients and offer regular monitoring.

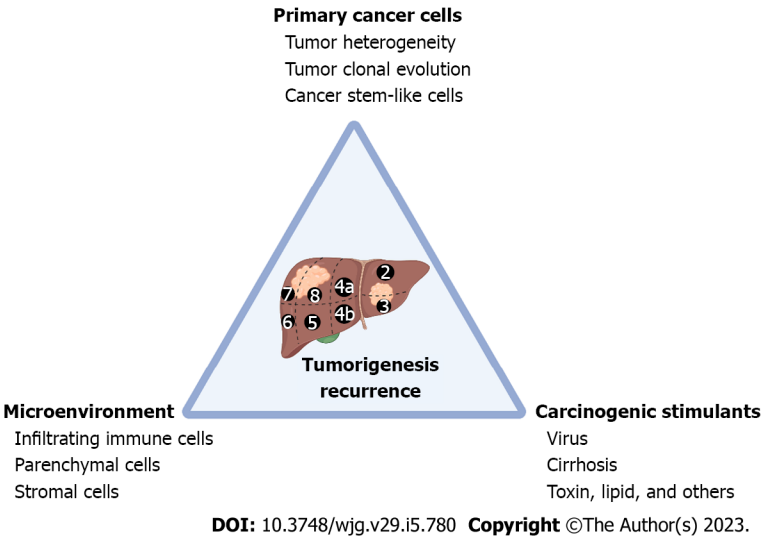
Traditionally, post-treatment surveillance of HCC utilized periodic cross-sectional imaging and tumor markers for patient follow-up[6]. The major aim of post-treatment surveillance is early identification of diseases that might be amenable to subsequent local therapy[7]. However, multiphase, contrast-enhanced computed tomography and magnetic resonance imaging suffer from low per-lesion sensitivity[8], difficulty in assessing small HCCs[9], and post-locoregional therapy lesions[10,11]. Ancillary methods like alpha-fetoprotein (AFP) and des-gamma-carboxy prothrombin likewise suffer from high false positive and false negative results[12,13]. The imperfection of early HCC recurrence detection urges the need to seek a more reliable armamentarium.

In addition, owing to the inclination to the multifocal occurrence of HCC, it is often challenging to decipher whether the lesions observed after curative liver resection arise from primary HCC or multicentric origin. Differentiation of the two types of multifocal lesions is necessary since their distinct mechanisms may have different effects on the response to treatment[14,15]. Some researchers attempt to distinguish the two conditions based on temporality of tumor occurrence. Early intrahepatic recurrence (IHR), defined as recurrent tumors detected within fewer than 2 years after surgery, has been attributed to residual lesions or intrahepatic micrometastasis from the initial tumor, while late (more than 2 years after surgery) IHR is largely considered a newly developed primary lesion[16,17]. However, such a definition has limited diagnostic accuracy and has not been validated[14,18].

Early studies have analyzed the clonality of multiple HCCs by assessing DNA ploidy, hepatitis B virus (HBV) integration sites, or microsatellite aberration mainly involving loss of heterozygosity and copy number variations (CNV)[19]. The heterogeneity not only exists among multifocal tumors but has also been found within a single lesion[20]. The molecular technologies that have been utilized for these approaches, such as DNA fingerprinting and whole-exome sequencing, are beyond the scope of this review and therefore will not be discussed further. We aimed to emphasize the complexity and heterogeneity behind recurrence widely seen in clinical practice.

The incidence of IHR of HCC after curative resection may be influenced by central factors including the specifics or clonality of primary tumor cells, the microenvironment that offers a susceptible niche for tumor cells to metastasize, and the existence of distinct carcinogens[21,22]. Reviewing past literature, we propose a model of three causations to illustrate the interplay between the factors that determine the recurrence of HCC, analogous to the well-known epidemiologic triangle for infectious disease (Figure 1). The recurrence of HCC results from interactions between primary cancer cells, the tumor microenvironment, and carcinogenic stimuli.

In recent years, transcriptome analysis emerged as a powerful tool to investigate the expression of disease phenotype and its association with genotype[23-25]. The evolution of bench work and laboratory equipment enables assays to be more efficient, enjoy higher throughput, and be more cost-effective. Such advances in molecular biological technology have facilitated the investigation of three causative domains of HCC recurrence described above and yielded abundant results. In the current



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Figure 1 Causal model of hepatocellular carcinoma recurrence. The figure was created with BioRender.com.

review, we focused on describing the role of transcriptome analyses, including real-time quantitative reverse transcription (RT-q) PCR, microarrays, and RNA sequencing (RNA-seq), as well as the rapidly evolving single-cell transcriptome analysis, among the latest work on this topic. We summarized the major findings of studies that may provide us with a clearer picture of HCC recurrence and give us insight into potential diagnostic targets as well as therapeutic strategies.

QUANTITATIVE RT-QPCR ANALYSIS OF RECURRENT HCC

Kary Mullis invented PCR in 1984. Russel Higuchi and colleagues later exploited fluorescence technology, making it possible to monitor PCR results using fluorescent probes[26,27]. These advances, combined with reverse transcriptase, which had been discovered earlier in 1970, brought about the development of RT-qPCR[28]. Since the late 1990s, RT-qPCR has been widely utilized in the exploration of differential gene expression (DGE) in various diseases, including HCC.

Several studies have utilized RT-qPCR to analyze the DGE in HCC recurrence. To clarify the genes responsible for the hematogenous spreading of HCC cells, one study measuring the expression of matrix metalloproteinase 9 (*MMP9*) and vascular endothelial growth factor in pairs of non-tumor and tumor samples with RT-qPCR found that the expression of *MMP9* in tumors was related to recurrence, while the expression of vascular endothelial growth factor was not. The same study also examined *AFP* mRNA in blood samples and found that the level was associated with recurrence and could serve as a predictor of recurrence or metastasis of HCC[29]. Similarly, another study reported that the mRNA level of *AFP* in peripheral blood samples significantly correlated with postoperative extrahepatic metastasis and disease-free survival[30].

In RT-qPCR, the quantification of complementary DNA (cDNA) from genes of interest is typically compared to that of reference genes, also called housekeeping genes, to allow the normalization of differences seen in different samples. Common reference genes include beta-actin (*ACTB*), beta-2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), hypoxanthine phosphoribosyltransferase 1, and TATA box binding protein[31]. A suitable reference gene must have stable expression across different conditions of samples. It is noteworthy that both *ACTB* and *GAPDH*, two commonly used reference genes, have been reported to be highly expressed in HCC when compared with non-tumor tissues[32], while TATA box binding protein and hypoxanthine phosphoribosyltransferase 1 were reported to be more suitable reference genes in HCC[33]. However, many currently available data with detection of significant DGE of HCC still used *ACTB* and *GAPDH* as reference genes[34,35], and whether the two genes are reliable for RT-qPCR normalization in HCC specimens requires more investigation.

The step of cDNA amplification endows RT-qPCR with a wide dynamic window and relatively high sensitivity to detect genes expressed with low abundance such as cytokines, and RT-qPCR is regarded as the "gold standard" of transcriptome analysis[36]. However, RT-qPCR also carries several limitations. Technically, various factors may impact the amplification and cause deviation from the ideal mathematical model of PCR, including the RNA quality, the efficiency of RNA-to-cDNA conversion, the primer quality, operator technique, as well as the "Monte Carlo" effect, an inherently and unavoidably high variance in the results from PCR reactions with a low starting template concentration[37]. In terms of its application in transcriptome analysis of clinical diseases, it is relatively low-throughput and can only

test a limited number of genes of interest with known sequences[38].

These limitations largely confine its ability to discover novel DGE of disease status. Only when researchers already know which "suspect" genes or pathways to target can RT-qPCR efficiently identify the disease-related DGE. Without predefined genes of interest and sufficient biological plausibility, it is challenging to identify novel disease-specific DGE solely with RT-qPCR. To achieve the prospective profiling of the transcriptome of HCC and recurrence, a more high-throughput technology that is capable of screening massive numbers of genes with various degrees of probability to have a disease-specific expression in parallel is needed. Thus, in the research of HCC, RT-qPCR is mainly used as a validation tool to confirm the DGE identified by two other technologies that emerged later on: microarrays and RNA-seq. These two research tools have yielded abundant results in all three aspects of HCC recurrence in the causal model we propose (Figure 1).

MICROARRAY ANALYSIS OF RECURRENT HCC

First developed by Schena *et al*[39] at Stanford University in 1995, microarrays have been widely applied in medical research as a high-throughput tool to reveal gene expression in disease status[39]. Microarrays can be divided into two main categories: cDNA microarrays and oligonucleotide microarrays. The surfacing of commercial platforms, such as Human UniGene Set RZPD 1 clone set for cDNA microarrays and Affymetrix Human Genome U95Av2 array for oligonucleotide arrays, have made the technology for both types of arrays widely accessible[40]. Additionally, the oligonucleotide microarrays have further been developed to identify single nucleotide polymorphism (SNP), named SNP arrays, in which the probes designed for harboring the SNP positions are hybridized with fragmented DNA molecules to examine the specific alleles of all SNPs[41]. The advance in microarray technology allows researchers to screen tens of thousands of RNA transcripts simultaneously and makes it possible to identify new genes with DGE related to diseases or specific pathophysiological conditions of interest. In addition to its high-throughput applicability, it has other major advantages including the wide availability of uncomplicated bioinformatics tools, more manageable data, and relatively low cost[38].

Microarray analysis has long played a central role in the field of HCC research. Iizuka *et al*[18] conducted a comprehensive review of the abundant data yielded with this revolutionizing technology [18], in which the authors classified the microarray-based approaches into three groups based on the distinct objectives of the studies, class comparison, class discovery, and class prediction, as proposed by Simon *et al*[42]. Such classification, however, was mainly according to study methodologies but hardly compared findings in the context of biological mechanisms and pathogenesis of HCC. To organize the diverse findings of microarray analyses in an integrated manner and to provide insights with pathobiological plausibility, we summarized the currently available data within the framework of our causal model of HCC, including the carcinogenic profile of primary cancer cells, carcinogenic stimuli, and the tumor microenvironment, in the following paragraphs.

Carcinogenicity of primary HCC cells

Lau *et al*[32] utilized cDNA microarrays to analyze the differential expression of mRNA of 4000 genes in paired HCC and noncancerous tissues[32]. They found that 211 genes were upregulated while 147 genes were downregulated, of which six genes were highly expressed and ten genes were downregulated in more than 30% of pairs. This was the first time when microarray technology was used in humans. Subsequently, either cDNA or oligonucleotide microarray studies, targeting either cell lines or patient-derived samples, have been widely conducted to discover the DGE related to the carcinogenic profile of primary HCC cells. The differentially expressed genes that have been identified include those associated with cell-cell interaction[43,44], transcription factors[18,43,45,46], apoptosis[43,45,47], cytokines[43,45], growth factors and/or growth suppression signals[43,45,48,49], cell proliferation[44,45,47,49,50], the cell cycle[43,45,49], tissue-specific expression proteins related to cell differentiation and development[45, 51], metabolism[49], angiogenesis[43,45,49], and stress-related response[35,50]. With the maturity and extensive application of microarrays, researchers further exploit this powerful technology to identify genes associated with HCC progression and prognosis. For instance, one study found that the upregulation of *ADAR, PSMD4, D9SVA, CCT3, GBAP, RDBP*, and *CSRP2* with downregulation of *IL7R* were associated with dedifferentiation of HCC[52]. Other studies focusing on metastasis and rapid progression of HCC identified differential expression in vimentin[53], granulin-epithelin precursor[54], ephrin-A1[53], and N-Myc downregulated gene 1[55].

It has also been widely known that differential expression of certain genes in primary cancer cells is associated with early recurrence. One study identified the expression profile of claudin-10, along with the pathological tumor-node-metastasis (pTNM) stage, to be independent predictors for HCC recurrence, and the results were validated with RT-qPCR[56]. Another study compared DGE of patients with recurrence *vs* those without, and it was found that four HLA genes (*HLA-DRA, HLA-DRB1, HLA-DG*, and *HLA-DQA*) encoding major histocompatibility complex class II antigens had significantly lower expression in the early IHR group[57]. Furthermore, while the DGE detected by microarrays and RT-

qPCR, as well as pathological tumor-node-metastasis stage and venous invasion were all found to be associated with early IHR in the univariate association study, the multivariate study only identified DGE of *HLA* as an independent predictor for early IHR. Iizuka *et al*[58] compared the gene expression of tumor cells between patient groups with and without recurrence and found that cell adhesion-related genes, including *ITGA6* and *SPP1*, had higher expression levels in HCC with early IHR[58].

Given that portal vein invasion (PVI) is known as a major prognostic factor of HCC recurrence[16,59-61], many studies used microarrays to identify the DGE related to PVI. One study found that cell growth-related genes *TAF4B*, *SLC4A7*, *RAB38*, and *RYR1* were associated with PVI[62]. Another study discovered that upregulation of *MMP14* and downregulation of two cytochrome P450 enzyme (*CYP*) genes, *ADAMTS1*, and *ITGA7* were associated with PVI[63,64]. Moreover, one study identified DGE of 110 sequence tags, *RHOC*, and two small GTPase-related genes (*ARHGAP8* and *ARHGEF6*) to be PVI-associated[63,64]. Lastly, one of the studies listed above successfully used PVI-associated DGE data from the microarray analysis to predict recurrence after surgical resection of HCC[62]. Although not directly analyzing recurrence-associated DGE, these studies offered abundant insight into the carcinogenicity of primary cancer cells defined by PVI.

Knowledge established by microarray studies has commonly been combined and applied to predict the recurrence and outcome of HCC. A data mining study examining the DGE between patients with IHR and those without recurrence based on pre-existing microarray databases generated one set of four differentially expressed genes (*STC1, FOXK2, MMP1*, and *LOXL2*) that promoted either cell cycle advancement or histone modulation could predict the incidence of early recurrence[65]. Another study conducting microarray analysis in human primary HCC tumors developed a 172-gene molecular prediction system for early IHR and tested its performance in independent cases[66]. The value of the predictive system was found to be a significant prognostic factor according to multivariate Cox regression analysis. Thus, DGE related to early IHR can be designed to predict clinical outcomes.

In summary, identification of DGE using microarrays, either by directly comparing recurrence with non-recurrence groups or indirectly looking at surrogate predictors such as PVI, enables us to clarify the carcinogenicity and the propensity of recurrence in primary HCC.

Carcinogenic stimuli

Clinical association studies have identified various risk factors of HCC. Common risk factors for HCC include HBV and hepatitis C virus (HCV) infection, cirrhosis, alcoholic liver disease, and nonalcoholic fatty liver disease. Less common risk factors include exposure to environmental toxins, Wilson's disease, hereditary hemochromatosis, alpha1-antitrypsin deficiency, primary biliary cholangitis, and autoimmune hepatitis[67]. In addition to the carcinogenicity of the primary HCC cells, these carcinogenic factors also play a decisive role in the development and recurrence of HCC. Microarray studies with clustering analysis based on some of these clinicopathological features have been widely performed to provide information about how risk factors contribute to HCC at a molecular level, with the greatest proportion of data coming from studies related to viral hepatitis.

Some studies compared the DGE of HCC cells to that of noncancerous liver tissues in HBV-positive and HCV-positive groups[67,68]. One study directly used oligonucleotide microarrays to compare transcriptomes in HBV-associated *vs* HCV-associated HCC, finding DGE in 83 genes, of which 31 and 52 genes showed increased expression in HBV and HCV-associated HCC, respectively[69]. The genes with DGE found in HBV-positive HCC mainly involved imprinted genes and genes associated with signal transduction, transcription, and metastasis, while in HCV-positive HCC the DGE was mainly found in genes related to detoxification or immune response. Such findings highlight the distinct mechanisms of viral carcinogenesis.

Another study used cassette ligation-mediated PCR to identify the human genome sequence next to the HBV DNA integration site and then conducted a microarray experiment to directly measure the characteristic expression of the affected genes[70]. In addition to viral hepatitis, certain toxins are also known as carcinogenic in favor of HCC, and microarray technology has been utilized to predict the carcinogenicity of chemicals by analyzing changes in gene expression in animal or cell culture models [71,72].

Finally, cirrhosis of any etiology is a major risk factor in the development of HCC. One study compared the gene expression profiles between HCC in patients with cirrhosis and without cirrhosis and identified several genes related to the regulation of inflammation, growth, and invasion of precancerous cells in cirrhotic liver, including C-C motif chemokine receptor and ligand (*CCR7* and *CCL5*), C-X-C motif chemokine ligand, and cytochrome P450 enzymes (*CYP2E1*, *CYP2C9*, and *CYP2A6*)[73].

In terms of recurrence, Kim *et al*[74] developed a risk scoring system with DGE of 65 genes identified with microarrays analyzing primary tumor cells in HBV-positive HCC patients and validated this system in another group of patients, finding that the classifiers successfully predicted early recurrence but failed to predict late recurrence[74]. The same author group further tried another approach by conducting a systemic analysis of gene expression from non-cancerous human liver tissue undergoing hepatic injury and regeneration. They identified a 233-gene signature that was significantly associated with late recurrence of HCC and validated the system in HBV-positive HCC patients who had received curative surgical treatment[75]. Network analysis of the gene signature identified signal transducer and activator of transcription 3/Notch signaling activation to be significantly related to late recurrence of

HCC. With prediction using microarray and multivariate logistic regression analysis, the authors scaled down the system to a four-gene (*RALGDS, IER3, CEBPD,* and *SLC2A3*) model that could successfully predict HCC recurrence. Interestingly, different models targeting cancerous or non-cancerous tissues could predict early and late recurrence of HCC, respectively, which may reflect the distinctive pathogenesis behind early and late recurrence of HCC in HBV-positive individuals.

Similarly, the recurrence of HCV-positive HCC has also been well studied. One study compared the DGE of noncancerous liver tissues from HCV-positive HCC patients with single nodular HCC recurrence and multicentric recurrence. Next, the authors developed a predictive system based on DGE in 36 genes, which was validated to successfully predict multicentric recurrence[76]. Also targeting HCV-positive HCC, another study found that the DGE profile observed in primary HCC biopsy or explant could not predict recurrence-free survival, while those yielded from noncancerous tissues could [77,78], which agreed with other studies on HBV-positive HCC[74,75]. Therefore, regardless of HBV or HCV-positive HCC, late recurrence was more likely to originate from a new clone of cells rather than the original HCC cells.

Another study focusing on genes associated with recurrence of HCC in HCV-positive patients awaiting liver transplantation identified the DGE profile of genes related to viral response as well as transcriptional network regulated by interferons, specifically interferon-α/β-inducible genes (signal transducer and activator of transcription 1, *OAS1*, and *MX1*), to be associated with recurrence-free survival[78]. The study also found that *FAIM3*, an anti-apoptotic gene, and *USP18*, a gene encoding an enzyme of the deubiquitinating protease family, were overexpressed in patients with recurrent HCC. Collectively, these studies exemplified how microarrays contribute to our understanding of carcinogenic stimuli in HCC recurrence.

Tumor microenvironment

Compared to the other two components in our causal model of HCC recurrence, microarray-based studies focusing on the tumor microenvironment and surrounding tissue suffer from less available data. This may be due to the limited ability of microarrays, as one of the "bulk" transcriptome analysis methods, to reveal the status of an individual cell. However, we found one study using cDNA microarrays to compare the gene expression profiles of noncancerous peripheral tissue from two HCC patient groups: those with primary HCC and venous metastases or confirmed extrahepatic metastases by follow-up, termed metastasis-inclined microenvironment samples; and those with HCC without detectable metastases, termed metastasis-averse microenvironment[79]. The authors found DGE in *HLA-DPA1*, *HLA-DRA* (antigen-presenting dendritic cells, B cells, epithelial cells), *PRG1*, and *ANXA1* to be associated with a metastatic phenotype, reflecting a T helper 2-predominant, anti-inflammatory cytokine profile, for which *CSF1* may be responsible. Interestingly, the increased expression of *HLA-DRA* in surrounding noncancerous tissue, as observed in this study, contrasts with one of the studies we cited above in which *HLA-DRA* genes showed lower expression in tumors[57]. The spatial distribution, migration of immune cells, and dynamic nature of the microenvironment may serve as plausible reasons for the discrepancy.

RNA-SEQ ANALYSIS OF RECURRENT HCC

Although microarray technology allows simultaneous measurement of numerous genes in one sample, it still has major drawbacks such as the limited dynamic range, high background noise, and an inability to detect novel transcripts. The advent of next-generation sequencing technology gave rise to a new technology, RNA-seq, that can address these limitations.

In 2008, RNA-seq was initially described by Nagalakshmi *et al*[80] as a new quantitative sequence-based method to map transcribed regions of the yeast genome[80]. Compared to microarray-based methods, RNA-seq enjoys higher genome coverage and better profiling of dynamic transcriptomes, providing information about alternative splicing, allele-specific expression, non-coding RNA, and SNPs. Unlike RT-qPCR and microarrays, which are largely limited by the requirement for *a priori* knowledge of the sequences being interrogated, RNA-seq is exclusively competent to discover novel transcripts [81]. As a result, despite disadvantages such as higher cost and large dataset generation, RNA-seq has been replacing microarrays over the past decade for transcriptome analyses in basic and translational research[82]. A comparison of RT-qPCR, microarrays, and RNA-seq are summarized in Table 1. The typical workflow for RNA-seq and microarray analysis in HCC translational research is illustrated in Figure 2.

Carcinogenicity of primary HCC Cells

RNA-seq enables us to identify specific DGE and expand our knowledge of the pathogenesis of HCC. One study performed pairwise DGE analysis between HCC and non-HCC tissues, finding upregulation of oxidative phosphorylation and higher expression of associated DNA damage-related signals in HCC compared to non-HCC samples. These findings suggest development of HCC may result from oxidative stress generated from overactive oxidative phosphorylation[83]. Another study analyzing poorly differ-

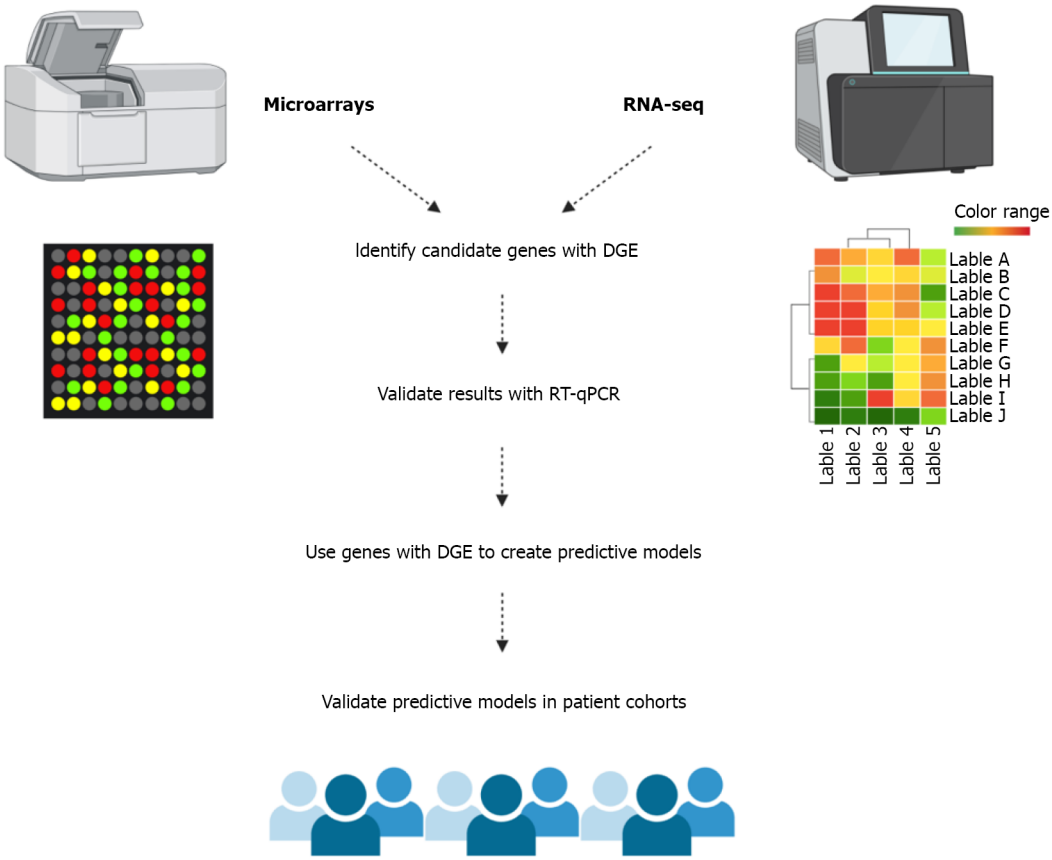
Table 1 Comparisons of real-time quantitative reverse transcription PCR, microarrays, and RNA-sequencing and their applications in hepatocellular carcinoma recurrence

	RT-qPCR	Microarrays	RNA-seq
Basic steps	RNA isolation, genome DNA removal	RNA isolation, mRNA extraction	RNA isolation, mRNA extraction
	cDNA preparation with RT	cDNA library preparation	Quality and quantity check
	Use of primers for amplification	Labeling with fluorescence	cDNA library preparation
	Data analysis	Hybridization with transcript probes on slides	Sequencing
		Scanning	Data analysis
		Image processing and data analysis	Validation
		Validation	
Throughput	Low	High	High
Dynamic range/sensitivity	Widest/high	Narrow/low	Wide (compared to microarrays)/high
Need for reference genome	No	No	Yes
Known sequences of genes of interest	Required	Required	Not required
Cost	Low	Low	High
Advantages	Low cost, simple	High throughput	Ability to detect novel differential transcripts
	Highest dynamic range	Relatively low cost	Splice junctions, SNP, non-coding RNA
	Gold standard	Good bioinformatics and statistical practices	
Downsides	Dependence on pre-existing knowledge of genes of interest	Difficulty to detect novel transcripts, non-coding RNA, splicing, or other dynamic natures of transcriptome	Large data storage
	Low throughput		High cost
		Need for designing probes	
		Low dynamic range	
Applications and main achievements in HCC recurrence-related research	Commonly used as a validation tool for confirming DGE results yielded from other high throughput analyses[56]	Providing abundant information on carcinogenicity of primary HCC cells and carcinogenic stimuli; laid the foundation for our current understanding of the pathogenesis of HCC recurrence[18]	Prospectively discovering DGE as potential novel classifiers for the carcinogenic profile of recurrent HCC cells; elucidating how HBV triggers HCC recurrence by interrupting the human genome[92,94,96]

cDNA: Complementary DNA; DGE: Differential gene expression; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; RNA-seq: RNA-sequencing; RT: Reverse transcription; RT-q: Real-time quantitative reverse transcription; SNP: Single nucleotide polymorphism.

entiated, moderately differentiated, and well-differentiated HCC with RNA-seq found DGE in poorly differentiated HCC to be mostly associated with cell metabolism, cell cycle, translation, and blood coagulation, of which the upregulation of *NOVA1*, *NSMCE2*, and *KIAA0196* and downregulation of *AQP9* were validated with RT-qPCR[84].

Since RNA-seq has a greater dynamic range and is more capable of detecting genes expressed with low abundance, another study analyzed blood samples taken from HCC and non-HCC patients in which the authors first identified 1578 dysregulated genes with RNA-seq and then validated them with RT-qPCR. Six genes (*SELENBP1, SLC4A1, SLC26A8, HSPA8P4, CALM1,* and *RPL7p24*) were differentially expressed, and the *CALM1* expression level was found to decrease along with tumor enlargement and thus had potential as a novel biomarker for tracking HCC[85]. While RT-qPCR also has a wide dynamic window and has been used to analyze peripheral blood during diagnosis[29,30], the ability of RNA-seq to scan many genes at a whole-genome scale makes the identification of candidate biomarkers more efficient. Moreover, RNA-seq is exclusively capable of analyzing non-coding RNA and has been utilized to identify long non-coding RNA (lncRNA) as well as small nucleolar RNA host genes. For instance, *SNHG4*, which is involved in the regulation of ribosomal RNA synthesis, RNA processing, and surveillance pathway, was found to be closely related to the tumorigenesis of HCC[86]. These studies



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Figure 2 Typical workflow of bulk transcriptome analysis in translational hepatocellular carcinoma research. The figure was created with BioRender.com. DGE: Differential gene expression; RNA-seq: RNA-sequencing; RT-q: Real-time quantitative reverse transcription.

exemplified the unique advantages of RNA-seq in discovering cancer mechanisms.

RNA-seq has also been used to analyze the carcinogenicity of HCC cells specifically related to recurrence. One study performing transcriptome analysis of 128 post-liver transplant HCC recurrence tissue samples found that the DGE was mainly found in genes involved in DNA synthesis, chromatin segregation, and mitosis, which might facilitate DNA replication and the growth of cancer cells[87]. The authors also performed mutation analysis in this study. Interestingly, the expression of some well-known mutations previously identified in HCC, such as p53, beta-catenin 1, and telomerase reverse transcriptase, did not appear to be significantly associated with HCC recurrence or prognosis. One explanation the authors proposed was that tumors recur after circulating HCC cells present at the time of transplantation traverse through the circulation, survive the turbulent flow environment, proceed through the pulmonary circulation, and finally seed themselves within the new liver. Another possible explanation is that recurrence is influenced by a complex interplay between primary cancer cells and "extratumoral" factors such as neurotransmitters, metabolism, or other constituents. This explanation is consistent with previous results from microarray studies finding that DGE of primary tumor cells failed to predict later recurrence[74,78].

Exploiting the merits of RNA-seq for detecting SNP and CNV, one study comparing liver gene expression in transplant patients with and without recurrence found glutathione S-transferase A2 (*GSTA2*) expression to be associated with early phase systemic injury and reactive oxygen species levels. Moreover, *GSTA2* could serve as a predictor of recurrence. Further, the authors identified that the G335C SNP of the *GSTA2* coding sequence, corresponding to an S112T amino acid substitution, was associated with HCC recurrence and survival[88]. Another study analyzing pairwise DGE analysis between primary and recurrent HCC found SNP variants of *GOLGB1* and *SF3B3* to be significantly related to more aggressive phenotypes[89].

Over the past decade, the popularity of high-throughput sequencing technology has not only made RNA-seq widely available but also brought about a publicly accessible comprehensive repository for genome-wide gene expression data such as the Gene Expression Omnibus and ArrayExpress. The Cancer Genome Atlas (TCGA) collects the results of cancer-related research, and mining of these shared databases provides insights by integrating results from different studies[90]. For example, Wang *et al* [91] identified a 77-gene signature associated with early HCC recurrence by conducting microarray experiments and cross-referencing the results with RNA-seq data from TCGA[91]. Combining data from

an ensemble of transcriptome profiling tools enables researchers to validate and complement results.

Carcinogenic stimuli

Thanks to its ability to capture the dynamic nature of transcriptome profiles, RNA-seq offers a unique advantage in investigating how carcinogenic factors alter transcriptome patterns in HCC development. It is known that HBV DNA can be integrated into the human genome and may result in somatic mutations[92]. One study found DGE of ten matched pairs in HBV-related HCC and non-HCC tissues to be mostly related to cell growth, metabolism, and immune-related pathways, which were significantly enriched at 8q21.3-24.3[93]. Moreover, the authors found a highly upregulated exon-exon junction at the *ATAD2* gene, an important protein that acts as a cofactor for Myc proteins, androgen receptor, and estrogen receptor-alpha. Also using RNA-seq, another study identified contrasting genomic and transcriptomic alterations such as HBV integration, somatic mutation, and CNV by comparing tumor with non-tumor samples[94]. For nonviral carcinogenic factors, one study identified 747 mRNAs and 8 lncRNAs with DGE between HCC and non-HCC cirrhotic tissues, narrowing down the results to 15 hub genes based on an association study with AFP levels in blood samples. Of these, *SPX*, *AFP*, and *ADGRE1* were validated in an independent HCC cohort[95].

With respect to recurrence, one study performed RNA-seq in HBV-related HCC patients to compare tumor and non-tumor tissues with various degrees of fibrosis[96]. HBV host genes overlapped with pathogenic SNPs in tumor suppressor genes of non-tumor tissues. Overlap was more significant in non-tumor tissues among recurrent cases, suggesting that tumor recurrence was highly associated with the integration of HBV genomes into precancerous tumor suppressor genes. Additionally, the difference in pathogenic SNP count between recurrent and non-recurrent patients was much larger in the low fibrosis group compared to the high fibrosis group, indicating that different recurrence risk models are needed for patients with low and high fibrosis. Taken together, these studies show the strength of RNA-seq in investigating the molecular genetic basis of HCC recurrence.

Tumor microenvironment

In addition to primary tumor cells and carcinogenic stimulus, researchers use this powerful tool to investigate the impacts of tumor microenvironment on recurrence. One study performing RNA-seq to analyze DGE between HCC tumors and surrounding cirrhotic tissue showed a gradual suppression of local tumor immunity coinciding with disease progression. In addition, the authors divided tumors into T cell-infiltrated and T cell-excluded based on the localization of CD8+ cytotoxic T lymphocytes visualized by immunohistochemistry staining and performed RNA-seq to examine the DGE between two groups. Twenty-three identified genes were associated with fibrosis and potentially modulated by transforming growth factor beta, platelet-derived growth factors, sonic hedgehog protein, or Notch pathways[97]. Given the emerging evidence suggesting that lncRNAs participate in cancer immunity, another study used RNA-seq to look at immune-related lncRNAs and related mechanisms from the TCGA database, identifying nine immune-related lncRNAs associated with HCC recurrence *via* Cox regression analysis[98]. The authors also created a recurrence prediction model based on their findings that was validated in an independent patient cohort.

The literature reviewed above shows how RNA-seq could be used to reveal molecular features of tumor immune biology in HCC progression and recurrence. However, most of the studies are limited to the discovery of DGE in bulk tissue samples, which may not be able to reflect the highly complicated tumor milieu and immune diversity. Although one study did consider tissue compartmentalization and tried to cluster cell populations to correlate DGE results with histology and immunohistochemistry staining[97], the approach was inefficient. The advent of single-cell RNA-seq (scRNA-seq) could address this issue and has improved our understanding of transcriptomes during cell-cell interaction. ScRNA-seq holds particular promise in research focusing on the tumor microenvironment, as described in the next section.

SINGLE-CELL TRANSCRIPTOME ANALYSIS OF RECURRENT HCC

Conventional bulk RNA-seq inherits the drawback of "averaging out" the data and does not have adequate resolution to delineate cell trajectory and cell-cell interactions. However, HCC is a heterogeneous disease with complex etiologies and tumor milieu[99,100]. There is thus a need to study the heterogeneity of tumoral cells and their ecosystem, particularly the immune cells.

To achieve a high resolution of cell subpopulations in malignant tissue, various single-cell isolation techniques have been developed. Generally, the isolation methods can be categorized into either cell marker-based selection or size-based selection, including fluorescence-activated cell sorting, laser microdissection, manual cell picking, serial dilution, magnetic-activated cell sorting, microfluidics, and CellSearch system[101,102]. When combining single-cell isolation with sequencing techniques, these technologies become promising tools to study intertumoral and intratumoral heterogeneity both spatially and temporally. Since mRNA transcriptome sequencing in a single cell was first reported in 2009[103], advances in sequencing techniques and single-cell selection methods have driven different

applications in the field of cancer biology. In recent years, droplet-based systems for high-throughput scRNA-seq such as inDrop, Drop-Seq, and 10X Genomics[104] have gained attention. Although previous studies used them to profile early HCC and its tumor microenvironment[105-107], studies for recurrent HCC are still limited.

Tumor heterogeneity, including intertumor and intratumor heterogeneity, is responsible for the recurrence of HCC[108]. Previously, researchers stressed the genomic profiling and molecular subclassification of intertumor heterogeneity[109-111]. Nevertheless, cancer cell adaptation, drug resistance, and tumor microenvironment are more closely related to intratumor heterogeneity[108]. To correlate the gene expression landscape of intratumor heterogeneity with HCC patient outcome, Losic *et al*[112] characterized a gene signature composed of 363 genes in the TCGA-HCC database[112]. The gene signature was associated with worse survival and was able to compete with other pre-existing single-biopsy prognostic signatures. The gene signature was also correlated with early tumor recurrence in the Heptromic Cohort as well as with higher levels of the prognostic biomarker AFP. At the single-cell level, the authors found transcriptional factor heterogeneity in the gene regulatory network by analyzing cis-regulatory sequence motifs from seven different locations in two HCC patients.

Another study found cellular heterogeneity in primary tumors similar to portal vein tumor thrombus and metastatic lymph nodes *via* scRNA-seq[113]. Additionally, authors focused on intratumoral T cells, in which they found CD8+ T cell clusters to be more enriched in HBV/HCV-related tumors compared to HBV/HCV-unrelated HCCs, concluding that chronic HBV/HCV infection may lead to CD8+ T cell exhaustion in HCC tumors. This phenomenon reflects the immune checkpoint blockade efficacy of viral-associated HCC in clinical scenarios, as the high checkpoint blockade response rate is related to CD8+ T cell density and programmed cell death protein 1 expression[114-116]. Furthermore, the authors found ligands highly expressed in protumorigenic and prometastatic hepatocytes related to inflammation (*e.g.*, C-X-C motif chemokine ligand 10/CXCR3) and immunosuppression (*e.g.*, macrophage-migration inhibitory factor/CD74), respectively. Distinct functions among malignant hepatocytes shape the immune microenvironment of HCC and provide hints to both tumor progression and immunotherapy.

Tumor recurrence and treatment resistance are partially determined by cancer stem-like cells (CSCs), which consist of a special subset of cells with stemness features and dictate cellular hierarchy and traits of dormancy and plasticity[117-119]. In a previous study, Zheng *et al*[120] combined transcriptome and functional analysis of HCC cells at the single-cell level to assess the degree of CSC heterogeneity as well as relationship to patient prognosis[120]. Discrete CSC subpopulations identified using single-cell surface markers all had a higher self-renewal ability compared to marker-negative cells but demonstrated appreciable biological differences in cell division and response to hypoxic stress in between. In addition, the authors found a 286-gene signature linked to CD133 and epithelial cellular adhesion molecule (EpCAM) are independent predictors of HCC patient survival. Moreover, HCC CSCs display an altered pattern of self-renewal heterogeneity when cultured under normoxia or hypoxia, suggesting a biological plasticity to these cells. Another article utilized scRNA-seq to identify two main HCC populations characterized by differential EpCAM expression[121]. Notably, a CD24+CD44+-enriched subclone within the EpCAM+ population exhibited a specific oncogenic expression signature and indicated the stemness of HCC. These findings were further confirmed by *in vitro* knockdown and *in vivo* tumorigenicity studies.

Sun *et al*[122] combined RNA-seq with single-cell profiling in paired samples from tumor and non-tumor regions of primary or recurrent HCC to unveil the unique immune ecosystem of recurrent HCC. The authors observed decreased regulatory T cells (Treg) and T cell proliferation with an increased proportion of CD8+ T cells and dendritic cells[122]. In addition, the authors concluded that CD8+ T cells in primary tumor and recurrent tumor samples showed the same transition trajectories but displayed considerably different immune and transcriptional states, suggesting that different immune therapy strategies should be considered for the treatment of primary and recurrent HCC. Specifically, CD8+ T cells in recurrent tumor samples, characterized by overexpression of *KLRB1*, revealed an innate dysfunctional state with low cytotoxicity and immunosuppressive phenotypes, which differed from the exhaustion state observed in primary tumor samples. The authors thus provided a model in which CD8+ T cell clones reside in a low proliferative and unresponsive state in the recurrent tumor due in part to tumor selection, suggesting that those cells are unable to recognize and eliminate recurrent tumor cells displaying subclonal neoantigens. In summary, data from this study indicated that malignant cells in the recurrent tumor demonstrated strengthened immune evasion capacities and reduced immune cell proliferation. Recurrent malignant cells could impair antigen presentation in dendritic cells *via* the programmed death-ligand 1-CD80 and cytotoxic T lymphocyte-associated protein 4-CD80 axes. Malignant cells may also recruit innate-like CD161+ CD8+ T cells *via* the CCL20-CCR6 axis, which could compromise anti-tumor immunity in early-relapse HCC.

Differentially expressed genes and pathway enrichment found in single-cell transcriptomes can be further applied to discover candidate drugs for the prevention of HCC recurrence as well as to the study of immune cell-cell communication. To predict the disease-free survival time and postoperative recurrence of HCC, Fu and Lei[123] constructed a risk score based on three immune cell types (effector memory CD8+ T cells, Treg cells, and follicular helper T cells) from the TCGA-HCC database called the T cell risk score[123]. Next, the authors used scRNA-seq data from 12 primary and 6 relapsed HCC samples to identify 645 genes with differential expression in three T cell types. After survival analysis,

the authors established a gene risk score by 15 prognostic genes (*AP000866.1, ATIC, CAPN10, EDC3, EID3, NCKIPSD, OXLD1, PHOSPHO2, POLE2, POLR3G, SEPHS1, SRXN1, TIMM9, ZNF487, and ZSCAN9*), which showed consistency with the T cell risk score in disease-free survival and immune characteristics. The results indicated these 15 hub genes may play a role in the process of immune cells affecting disease-free survival.

Subsequently, these hub genes were screened with CellMiner, a web tool based on the NCI-60 cell line set for identifying potential therapeutic drugs[124]. Pearson correlations between the 15 hub genes and the half-maximal inhibitory concentration of targeted drugs were analyzed, and the studies suggested that postoperative treatment of these drugs, such as imexon, irofulven, and nelarabine, may delay HCC recurrence. Moreover, the authors explored immune cell-cell interactions, finding the strongest communication among these three cell types was from effector memory CD8+ T cells to themselves *via* the granzyme A-coagulation factor 2 receptor signaling pathway as well as effector memory CD8+ T cells to follicular helper T cells and Treg cells *via* the CCL5-CCR4 signaling pathway. These findings illuminate crosstalk among such cell types, which is beneficial in future investigation of effector memory CD8+ T cells, Treg cells, and follicular helper T cells in disease-free survival time and recurrence prevention for patients with HCC.

LIMITATIONS AND FUTURE DIRECTIONS

The current healthcare system is transitioning from the time of evidence medicine towards the era of precision medicine. The omics technologies are evolving quickly and have built up tremendous results. Admittedly, there is still a gap to be bridged between the bench and bedside for translating these technologies.

First, the development of other levels of omics research (*e.g.*, epigenetics, proteomics, and metabolomics) expands cancer research including HCC to a multitude of data. How to incorporate and harness this huge amount of information and confirm its clinical importance (and not just an association) remains a challenge. With the advancement of computing power and artificial intelligence, we have the opportunity to store data and deal with multi-omics in parallel. Researchers can thereby extract significant data, confirm it with mechanistic studies in the laboratory, and translate it into clinical trials.

Second, studies showed that physicians who had lower confidence in genomic or transcriptomic technologies would like to ask for guidelines or training support[125-127]. To popularize transcriptome analysis into clinical practice, we require more physicians who understand the concepts of both omics technologies and tumor biology to stand out. Thus, we can improve medical education, design relevant clinical trials, and formulate health guidelines and policies. In fact, it is not merely a portion of people's responsibility. Multidisciplinary networks to share the collection of patient samples, clinical data, the standard of techniques, and genetic counseling are all indispensable to making medical decisions.

Last but not least, the accessibility of these technologies, especially the cost, is still a burden to patients. We hope in the future that these technologies can be applied more widely, and the price can be affordable for people by following "Moore's law" (*i.e.* higher throughput and lower cost) as in the computer industry (genome.gov/sequencingcosts; accessed November 19, 2022). Meanwhile, health policymakers should recognize the need for providing patients with transparent information and protecting their privacy. Therefore, we can benefit most patients by achieving the P4 discipline (preventive, predictive, personalized, and participatory) in precision medicine[128].

CONCLUSION

The high recurrence rate of HCC remains a serious burden to patients undergoing curative treatments and a major challenge to patient outcome. Over the past few decades, the emergence and evolution of transcriptome profiling methods have benefited the discovery of disease mechanisms, diagnosis, and treatment of HCC recurrence. Our current understanding of HCC pathophysiology is largely based on the fruitful results yielded by transcriptome analysis technology. We found that these abundant studies can be categorized based on the three domains of tumorigenesis, which include carcinogenic profile of primary cancer cells, carcinogenic stimuli, and tumor microenvironment.

We herein summarized the major findings of RT-qPCR, microarray, RNA-seq, and scRNA-seq research under the framework of these three domains (Table 2) and in doing so revealed the strengths and limitations of each technique. Being low-throughput, RT-qPCR is limited in identifying new DGE and is mainly used to validate findings yielded by other high-throughput techniques. Microarrays and RNA-seq have yielded great achievements in the study of carcinogenicity among primary cancer cells and carcinogenic stimuli. Moreover, these technologies contributed largely to our current knowledge on HCC recurrence pathogenesis. To date, a workflow consisting of microarray/RNA-seq search for differential transcripts, RT-qPCR confirmation, predictive model generation, and independent patient cohort validation has become a standard approach in basic HCC science. However, current diagnostic and

Table 2 Representative transcriptomic studies in recurrence of hepatocellular carcinoma

Ref.	Method	Sample comparison	Major findings	Featured research domain
Jiang *et al*[29], 2000	RT-qPCR	Nontumorous liver *vs* tumor samples; peripheral blood from HCC patients	*MMP9* in tumors was related to recurrence. mRNA of *AFP* in blood samples was associated with recurrence	Primary cancer cells
Morimoto *et al*[30], 2005	RT-qPCR	Peripheral blood and bone marrow samples from patients with HCC *vs* benign diseases	*AFP* mRNA level in blood, but not bone marrow, could be useful for predicting postoperative tumor recurrence	Primary cancer cells
Cheung *et al*[56], 2005	Microarray	HCC tumors from patients with post-OP recurrence *vs* without recurrence	*CLDN10*, along with the pTNM stage, were independent predictors for HCC recurrence	Primary cancer cells
Matoba *et al*[57], 2005	Microarray	HCC tumors from patients with *vs* without post-OP early (< 1 yr) recurrence	*HLA-DRA*, *HLA-DRB1*, *HLA-DG*, and *HLA-DQA* had significantly lower expression in the early IHR group	Primary cancer cells
Iizuka *et al*[58], 2006	Microarray	HCC tumors from patients with post-OP IHR *vs* EHR	46 cell adhesion-related genes, including *ITGA6* and *SPP1*, had higher expression levels in HCC with early IHR	Primary cancer cells
Ho *et al*[62], 2006	Microarray	HCC tumors from patients with *vs* without PVI	Differential expression of 14 genes related to the human melanoma gene family, cell growth, DNA glycosylation, and thrombin inhibitors, can be used to predict recurrence	Primary cancer cells
Chen *et al*[63], 2002	Microarray	HCC tumor and corresponding nontumorous tissue with *vs* without PVI	*ARHGAP8* and *ARHGEF6* were PVI-associated.	Primary cancer cells
Okabe *et al*[64], 2001	Microarray	HCC tumor from patients with *vs* without PVI	Upregulation of *MMP14* and downregulation of two *CYP* genes, *ADAMTS1*, and *ITGA7* were associated with PVI	Primary cancer cells
Okamoto *et al*[76], 2006	Microarray	Multicentric *vs* single nodular recurrent HCV-related HCC	36 marker genes were associated with multicentric recurrence and were used to develop a predictive scoring system	Carcinogenic stimulants
Mas *et al*[78], 2007	Microarray	HCV-related HCC from patients with *vs* without disease progression	Upregulation of *FAIM3* and *USP18*, and downregulation of *TFP1*, *HIST1H4E*, and *NRG1* were related to disease-free survival after curative treatment	Carcinogenic stimulants
Nagalakshmi *et al*[80], 2008	Microarray	MIM *vs* MAM	*HLA-DPA1*, *HLA-DRA*, *PRG1*, and *ANXA1* were associated with a metastatic phenotype (Th2-predominant), for which *CSF1* may be responsible	Microenvironment
Yoshioka *et al*[66], 2009	Microarray	HCC tumors from patients with multiple early (< 2 yr) IHR *vs* with DFS > 3 yr	Informative gene sets including *PPARBP*, *RREB-1*, *BCL2*, *HDAC1*, and *BIRC5* were yielded and used for a predictive model, which was validated in independent cases	Primary cancer cells
Kim *et al*[74], 2012	Predictive model construction using microarray database	DGE in 65 genes from pre-existing databases were used for a predictive model for early HCC recurrence and validated in independent HBV-related HCC cohorts	A risk scoring system with 65 differentially expressed genes identified from microarray data successfully predicted overall survival < 3 yr post-OP	Carcinogenic stimulants
Kim *et al*[75], 2014	Predictive model construction using microarray database	DGE of 233 HIR-related genes from preexisting databases were used for a predictive model for late HCC recurrence and validated in independent HBV-related HCC cohorts	Genes related to STAT3/Notch signaling activation were related to late (> 1 yr) recurrence of HCC. *RALGDS*, *IER3*, *CEBPD*, and *SLC2A3* were independent predictors of recurrence.	Carcinogenic stimulants
Nakagawa *et al*[65], 2021	Predictive model construction using microarray database	Validation of intrahepatic metastasis risk signatures created based on a preexisting microarray database in an independent patient cohort	*STC1*, *FOXK2*, *MMP1*, and *LOXL2* that promote either cell cycle advancement or histone modulation could predict the incidence of early recurrence	Primary cancer cells
Liu *et al*[87], 2022	RNA-seq	HCC tumors from patients with *vs* without recurrence	Most altered expression genes are related to DNA synthesis (*MCM8*, *MCM6*, *TOP2A*, and *CDC7*), chromatin segregation (*BUB1* and *CDC6*), and mitosis (*NDC80* and *PPP2R3C*)	Primary cancer cells

Ng *et al*[88], 2021	RNA-seq	Paired tumor tissues *vs* nontumorous tissues from HCC patients	*GSTA2* expression was associated with early-phase systemic injury and reactive oxygen species levels and could serve as a predictor of recurrence	Primary cancer cells
Lachmann *et al* [90], 2018	RNA-seq	Paired primary *vs* recurrent HCC tumor tissues	Mutations of *GOLGB1* and *SF3B3* are potential key drivers for the aggressive phenotype in recurrent HCC	Primary cancer cells
Okrah *et al*[97], 2018	RNA-seq	HBV-related HCC tumor *vs* distant nontumorous liver tissues	More HBV gene integrations correlated with a higher recurrence rate	Carcinogenic stimulants
Wang *et al*[98], 2021	Validation of RNA-seq database	HCC tumors *vs* matched cirrhotic tissues; CD8+ CTL-infiltrated *vs* T cell-excluded tumor tissues	Local tumor immunosuppression coincided with disease progression. Association was found between elevated fibrosis and the T cell-excluded immune phenotype	Microenvironment
Ho *et al*[99], 2021	Predictive model construction using RNA-seq database	Validation of recurrence-associated lncRNAs identified by regression analysis of TCGA database	9 immune-related lncRNAs were tightly associated with recurrence	Microenvironment
Zheng *et al*[120], 2018	scRNA-seq	CSC *vs* non-CSC populations defined by triple+ or triple− surface expression of CD133, CD24, EpCAM	286 signature genes linked to triple+ CSC could predict tumor recurrence in 240 HCC cases with multivariable Cox regression survival risk prediction analysis	Primary cancer cells
Sun *et al*[122], 2021	scRNA-seq	Tumors from primary *vs* early-relapse HCC patients	Decreased Treg and T cell proliferation with an increased proportion of CD8+- T cells and DC were found in early-relapse tumors compared to primary tumors. CD8+ T cells with overexpression of *KLRB1* revealed an innate dysfunctional state with immunosuppressive phenotypes in recurrent tumors	Microenvironment
Fu and Lei[123], 2022	scRNA-seq	Primary *vs* early-relapsed HCC samples	ScRNA-seq analysis of primary *vs* relapsed HCC identified 645 genes with DGE across three T cell types. Univariate and multivariate analysis identified 15 prognostic genes (*AP000866.1*, *ATIC*, *CAPN10*, *EDC3*, *EID3*, *NCKIPSD*, *OXLD1*, *PHOSPHO2*, *POLE2*, *POLR3G*, *SEPHS1*, *SRXN1*, *TIMM9*, *ZNF487*, and *ZSCAN9*)	Microenvironment

AFP: Alpha-fetoprotein; CSC: Cancer stem cell; CTL: Cytotoxic T lymphocyte; *CYP*: Cytochrome P450; DC: Dendritic cell; DFS: Disease-free survival; DGE: Differential gene expression; EHR: Extrahepatic recurrence; EpCAM: Epithelial cellular adhesion molecule; *GSTA2*: Glutathione S-transferase A2; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HIR: Hepatic injury and regeneration; IHR: Intrahepatic recurrence; lncRNA: Long non-coding RNA; MAM: Metastasis-averse microenvironment; MIM: Metastasis-inclined microenvironment; *MMP9*: Matrix metalloproteinase 9; post-OP: Postoperative; pTNM: Pathological tumor-node-metastasis; PVI: Portal vein invasion; RNA-seq: RNA-sequencing; RT-qPCR: Real-time quantitative reverse transcription; scRNA-seq: Single-cell RNA sequencing; STAT3: Signal transducer and activator of transcription 3; TCGA: The Cancer Genome Atlas; Th2: T helper 2 cell; Treg: T-regulatory cell.

monitoring guidelines are still based on conventional AFP and imaging assessments, having yet to incorporate data from transcriptome analysis.

Whether these gene classifiers can be applied clinically to improve diagnostic accuracy and help identify high-risk patient groups needs further confirmation from population-based studies in groups more representative of the patient population than a limited research cohort. ScRNA-seq, with its remarkably high resolution, outperforms other tools in exploring cell-cell interactions and the tumor microenvironment. Although currently available studies are limited compared to other transcriptome techniques, the high resolution and high-throughput features of scRNA-seq make it a powerful tool with great potential in investigating the tumor environment. In an era when immunotherapy is rapidly advancing, the prospect of being able to decipher the cancer immune ecology serves as a continuous incentive for future scRNA-seq studies in HCC recurrence, which may help us to verify and optimize the efficacy of novel treatments as well as facilitate the inclusion of precision medicine in managing HCC recurrence.

FOOTNOTES

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Country/Territory of origin: Taiwan

ORCID number: Chun-Cheng Chiang 0000-0001-8105-2512; Hsuan Yeh 0000-0002-4926-8433; Siew-Na Lim 0000-0001-6867-8449; Wey-Ran Lin 0000-0001-6078-1396.

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REVIEW

Impact of chronic liver disease on SARS-CoV-2 infection outcomes: Roles of stage, etiology and vaccination

Riccardo Nevola, Livio Criscuolo, Domenico Beccia, Augusto Delle Femine, Rachele Ruocco, Simona Imbriani, Maria Alfano, Angela Villani, Antonio Russo, Pasquale Perillo, Raffaele Marfella, Luigi Elio Adinolfi, Ferdinando Carlo Sasso, Aldo Marrone, Luca Rinaldi

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Riccardo Nevola, Livio Criscuolo, Domenico Beccia, Augusto Delle Femine, Rachele Ruocco, Simona Imbriani, Maria Alfano, Angela Villani, Raffaele Marfella, Luigi Elio Adinolfi, Ferdinando Carlo Sasso, Aldo Marrone, Luca Rinaldi, Department of Advanced Medical and Surgical Sciences, University of Campania "Luigi Vanvitelli", Naples 80138, Italy

Riccardo Nevola, Pasquale Perillo, Internal Medicine and Hepatology Unit, Ospedale Evangelico Betania, Naples 80147, Italy

Antonio Russo, Department of Mental Health and Public Medicine, University of Campania "Luigi Vanvitelli", Naples 80138, Italy

Corresponding author: Riccardo Nevola, MD, PhD, Doctor, Department of Advanced Medical and Surgical Sciences, University of Campania "Luigi Vanvitelli", Miraglia Place, Naples 80138, Italy. riccardo.nevola@unicampania.it

Abstract

Since the first identification in December of 2019 and the fast spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, it has represented a dramatic global public health concern. Though affecting mainly the respiratory system, SARS-CoV-2 disease, defined as coronavirus disease 2019 (COVID-19), may have a systemic involvement leading to multiple organ dysfunction. Experimental evidence about the SARS-CoV-2 tropism for the liver and the increasing of hepatic cytolysis enzymes during infection support the presence of a pathophysiological relationship between liver and SARS-CoV-2. On the other side, patients with chronic liver disease have been demonstrated to have a poor prognosis with COVID-19. In particular, patients with liver cirrhosis appear extremely vulnerable to infection. Moreover, the etiology of liver disease and the vaccination status could affect the COVID-19 outcomes. This review analyzes the impact of the disease stage and the related causes on morbidity and mortality, clinical outcomes during SARS-CoV-2 infection, as well as the efficacy of vaccination in patients with chronic liver disease.

Key Words: SARS-CoV-2 infection; COVID-19; Chronic liver disease; Cirrhosis; Non-alcoholic fatty liver disease; Liver injury

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Core Tip: It has been observed, since the early months of the pandemic, that pre-existing liver disease was associated with a worsening of clinical outcomes in severe acute respiratory syndrome coronavirus 2 infection. A correlation exists between severity of liver disease and coronavirus disease 2019-related adverse outcomes. The etiology of liver disease could significantly affect mortality rates, as well as vaccination status.

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INTRODUCTION

From December 2019, the fast spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a new virus belonging to the Coronavirus family of respiratory pathogens, has represented a major public health problem worldwide, leading to the declaration of a pandemic status in March 2020 by the World Health Organization (WHO)[1]. Despite the enormous efforts by health personnel and organizations, coronavirus disease 2019 (COVID-19) has caused more than 6.5 million deaths worldwide to date [2]. While the new virus variants show a milder clinical picture with predominant involvement of the upper respiratory tract, the most severe form of SARS-CoV-2 infection characterized by acute respiratory distress syndrome (ARDS) still represents an important cause of morbidity and mortality[3-5]. As observed since the first pandemic phases, organ involvement in COVID-19 is not limited to the respiratory tract, but can result in systemic disease with cardiovascular, renal, neurological and, last but not least, hepatic involvement. In particular, increases in the indices of hepatic cytolysis or cholestasis [mean as an increase of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TBIL), gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP) upper range value] or a more severe acute liver injury (ALI) [defined as ALT and/or AST over 3 × upper limit of normal (ULN) or ALP, GGT, and/or TBIL over 2 × ULN] can be found in 14%-53% of SARS-CoV-2 infection[6]. The mechanism of COVID-19-associated ALI is probably multifactorial, given the combination of direct viral cytopathic effect, cytokine-induced inflammatory damage, hypoxic and drug induced liver injury[7]. ALI has been demonstrated to be a predictor of unfavorable SARS-CoV-2 infection course[8,9]. High levels of AST and TBIL at hospital admission are associated with an increased risk of COVID-19 progression[10]. The coexistence of previous liver disease worsens the outcomes of COVID-19. In fact, if on one hand SARS-CoV-2 infection can determine liver injury[6], on the other hand chronic liver diseases (CLDs) are associated to immune system and hemostasis alterations[11] that are able to accelerate some pathogenetic mechanisms of SARS-CoV-2, as cytokine storm and prothrombotic state, affecting the outcomes significantly[12,13]. In particular, patients with liver cirrhosis are at a high risk of an unfavorable SARS-CoV-2 infection course, with significantly higher mortality rates than the general population[14,15]. The occurrence of ALI during COVID-19 in this population is an additional predictor of all-cause mortality[16]. The risk of adverse outcomes in patients with chronic hepatitis is still poorly understood. Moreover, the etiology of CLD, the disease stage, potential concomitant therapies (*e.g.*, immunosuppressive) and vaccination status could significantly impact the outcomes of COVID-19.

The aim of this review is to describe the role of liver disease during COVID-19, analyzing if and how much the stage of the disease and the related etiology can affect the SARS-CoV-2 infection outcomes and examine what is known on the clinical efficacy of vaccination in patients with CLD to date.

COVID-19 OUTCOMES AND LIVER DISEASE

Despite the prevalence of a pre-existing liver disease in COVID-19 patients appearing low (between 0.6% and 3.4%)[17-19] and not significantly related to the risk of contracting SARS-CoV-2 infection[20], the first studies showed that the presence of CLD was associated with an increase in both hospitalization rate and overall mortality[14]. In this regard, Singh *et al*[14] reported a relative risk (RR) for death in patients with pre-existing liver disease of 2.8 (95%CI: 1.9-4.0). Similarly, also Williamson *et al* [19] and Galiero *et al*[21] found a significant association between liver disease and COVID-19 mortality. A meta-analysis including about 25000 patients confirmed that CLDs were significantly associated with

more severe COVID-19 course [odds ratio (OR): 1.48; 95%CI: 1.17-1.87] and overall mortality (OR: 1.78; 95%CI: 1.09-2.93)[20]. Other studies instead did not show this association[22,23]. For example, Simon *et al*[22] reported that patients with CLD show an increased risk of hospitalization for COVID-19 but not an increased mortality. Furthermore, in a recent retrospective case-control study, patients with CLD did not show more need for invasive mechanical ventilation, as well as admission to intensive care unit (ICU) and overall mortality, compared to patients without liver disease[23].

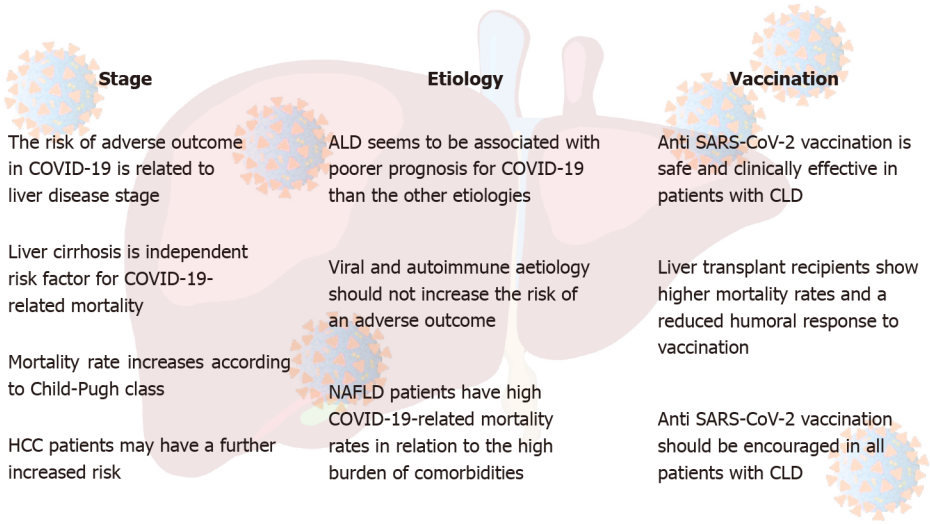
Therefore, data on the association between liver disease and increase of mortality rates related to COVID-19 are conflicting. However, the spectrum of CLDs is very heterogeneous both for etiology and for stage disease. In fact, the risk appears directly related to the latter. Studies that evaluated all liver disease stages could have been affected by different mortality rates in patients with CLD, greater in cirrhosis stage, particularly if in decompensation. In this regard, Mallet *et al*[15] more recently reported, on a large French cohort, a significant increase in the need for mechanical ventilation (OR: 1.54; 95%CI: 1.44-1.64) and mortality rate (OR: 1.79; 95%CI: 1.71-1.87) in patients with CLD. However, when stratified by disease severity, authors observed that patients with decompensated liver cirrhosis showed a significantly higher mortality rate whereas patients with mild liver disease or compensated cirrhosis were not at increased risk of COVID-19-related mortality[15]. These data have been confirmed by meta-analysis of Middleton *et al*[24], in which liver cirrhosis has been shown to be associated to an increased risk of all-cause mortality in COVID-19 compared to non-cirrhotic patients. Current evidences on the role of disease stage, etiology, and vaccination on COVID-19 outcomes are summarized in Figure 1.

LIVER CIRRHOSIS AND COVID-19 CLINICAL OUTCOMES

Regardless of the etiology, the stage of liver cirrhosis is characterized by a high degree of patient frailty. The impairment of immune system, the concurrent increase of both thrombotic and hemorrhagic risk and the protein-calorie malnutrition make the patient suffering from cirrhosis vulnerable to various kinds of injuries. The frailty of the cirrhotic patient in the setting of COVID-19 is expressed by an excess of mortality and hospitalization rate compared to patients without CLD[14,15,19]. Extracting data of the subgroup of cirrhotic patients, Singh *et al*[14] had already reported that the risk of death in these patients was a 4.6-fold increase compared to ones without liver disease. As already mentioned, the mortality rate appears higher in patients with cirrhosis not only compared to patients without CLD but also when compared to patients with CLD but without cirrhosis (32% *vs* 8%, respectively; $P < 0.001$)[25]. In particular, compared to cirrhotic/SARS-CoV-2 negative patients and to non-cirrhotic/SARS-CoV-2 positive patients, those with liver cirrhosis and COVID-19 had a 2.38- and 3.31-times adjusted hazard ratio of 30-d death, respectively[26]. Similar results were found by Ioannou *et al*[27]. Overall, the 30-d COVID-19-related mortality rate in patients with cirrhosis is very high, ranging from 32% to 47% of cases[25,28,29]. In fact, liver cirrhosis has been proven to be an independent risk factor for COVID-19 related mortality (OR: 3.1)[29]. However, no updated mortality data are available for the new viral variants, with an apparently lower lethality rate than the wild type. Although most studies consider liver cirrhosis as an independent predictor of the risk of COVID-19-related death[15,24,26,29,30], some data would indicate that the high mortality rates in patients with cirrhosis and COVID-19 result from cirrhosis-associated comorbidities and extrahepatic organ failure rather than the liver disease itself[31]. After propensity score matching for age, sex, and extra-hepatic comorbidities, mortality rate during COVID-19 appears to be similar between patients with and without cirrhosis (28.8% *vs* 26.1%, respectively; $P = 0.644$). These results still could have been affected by the typology of matching and the methodology of data collection through registers. Among patients with liver cirrhosis, the coexistence of obesity and diabetes would further worsen the outcomes[16].

Similarly, to what was recently reported for kidney failure[32], COVID-19-related mortality risk was strongly associated with the stage of liver impairment. Overall, the 30-d mortality risk is significantly increased in patients with decompensated liver cirrhosis hospitalized for COVID-19[15,30]. Mortality rates increased according to Child-Pugh (CP) class, raising from 19% of class A (OR: 1.90), to 35% of class B (OR: 4.14) up to 51% of class C (OR: 9.32)[25]. In particular a CP score ≥ 9 at hospital admission would be predictive of high mortality[16]. Compared to patients without liver disease, a CP class B and C cirrhosis would bring an additional mortality rate of +20% and +38.1%, respectively[25]. In this regard, it seems that the Chronic Liver Failure Consortium had better performance in predicting 28-d mortality than CP score and model for end-stage liver disease-Na in patients with cirrhosis and COVID-19[29]. Moreover, in cirrhotic patients, an increasing trend of bilirubin and AST/ALT ratio[16] or the occurrence of liver injury[10,21] would be predictive of mortality.

The most frequent cause of mortality in patients with liver cirrhosis and COVID-19 remains the respiratory failure (71%)[25]. However, acute liver decompensation occurred in up to 46% of patients, even without respiratory symptoms. Liver related complications increased according to stage of liver disease[16]. It is known that infections (bacterial more than viral) may lead to liver decompensation, hepatorenal syndrome and portosystemic encephalopathy, and are one of the most frequent causes of acute-on-chronic liver failure (ACLF) and death in patients with cirrhosis. Conclusive data on the comparison between the SARS-CoV-2 infection outcomes and those of other infectious precipitants are



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Figure 1 Impact of chronic liver disease on severe acute respiratory syndrome coronavirus 2 infection outcomes: role of stage, etiology and vaccination. ALD: Alcohol-related liver disease; CLD: Chronic liver disease; COVID-19: Coronavirus disease 2019; HCC: Hepatocellular carcinoma; NAFLD: Non-alcoholic fatty liver disease; SARS-CoV-2: Severe acute respiratory syndrome-coronavirus 2.

currently not available. However, some data indicate that in-hospital mortality rates would be significantly higher in cirrhotic patients with COVID-19 than in those with other bacterial infections [28]. Overall, about 45% of patients with CLD develop ACLF[16]. Higher rates are reported for patients with liver cirrhosis. Moreover, cirrhotic patients with diabetes or obesity had higher ACLF rates than non-diabetic or normal weight patients (OR: 2.1 and 8.9, respectively)[16]. Similarly, to other viral infections, ACLF during COVID-19 could result from an immune-mediated response to viral antigens in the context of a cytokine storm[33], as well as a direct cytopathic effect or iatrogenic injury. Multi-organ damage caused by SARS-CoV-2 is significantly more frequent in immunocompromised patients[34]. The impairment of the immune system in the case of liver cirrhosis due to bone marrow suppression, lower protein synthesis and cirrhosis associated immune dysfunction syndrome, could explain the high rates of ACLF and the severe course in cirrhotic patients.

If in-hospital, COVID-19-related mortality was significantly increased in patients with liver cirrhosis and little is known about post-acute outcomes. Recently, Vaishnav *et al*[35] analyzed the post-discharge mortality of cirrhotic patients with SARS-CoV-2 infection. The data indicate that mortality rates within 2 mo of discharge among COVID-19 survivors are comparable between patients with liver cirrhosis and those without.

Hepatocellular carcinoma

Little data is currently available on SARS-CoV-2 infection clinical course and outcomes in patients with hepatocellular carcinoma (HCC). Indeed, in studies performed during the first pandemic phase on COVID-19-related outcomes in cancer patients, those with HCC were underrepresented[36,37].

Although data are not univocal[25], several studies include the presence of HCC among the independent predictors of COVID-19-related mortality[15,30,38]. Among patients with CLD and COVID-19, HCC patients had 3.31 times the hazard of death for all causes, regardless of the presence of liver cirrhosis[30]. Beyond the association with mortality, according to Mallet *et al*[15] the presence of HCC is also predictive of a severe course of COVID-19 and a greater need for mechanical ventilation. Muñoz-Martínez *et al*[38] evaluated the SARS-CoV-2 infection course in 250 patients with primary liver cancer (218 with HCC and 32 with intrahepatic cholangiocarcinoma). In patients with HCC, a 30-d mortality rate of 18.4% was observed, with a statistically significant trend according to the stage of cirrhosis (assessed by CP) and tumor [assessed by Barcelona Clinic Liver Cancer (BCLC)]. In particular, the mortality rates increased from 6.10% for BCLC-0/A, to 11.76% for BCLC-B, to 20.69% for BCLC-C and 34.52% for BCLC-D[38].

The high COVID-19 related mortality in patients with HCC could result from the link between viral infection and the impairment of the immune-system secondary to active neoplasm, antineoplastic therapy and the frequent coexistence of liver cirrhosis.

Liver transplant recipients

In the analysis of the correlation between SARS-CoV-2 infection and CLD, patients who have undergone liver transplant (LT) represent a separate group due to the effects of chronic immunosuppressive therapy. The hypothesis that this therapy could increase the susceptibility to SARS-CoV-2 has been

suggested from some population studies[39-41]. Observational studies on western populations have reported a mortality of 16%-22% in hepatotransplant patients with SARS-CoV-2 infection[42,43] in liver-transplant patients with SARS-CoV-2 infection, finding an increased survival in the short-term LT recipients (< 2 years), usually treated with full doses of immunosuppressants. This data support the hypothesis that, more than the immunosuppressive effect itself, the main cause of death in these patients is represented by the long-term cardio-metabolic effects induced by immunosuppressive drugs [44].

The study of the correlation between the type of immunosuppressive drug and COVID-19 outcomes in patients who have undergone LT has led to non-univocal results. During the first pandemic wave, Colmenero *et al*[39] reported that mycophenolate mofetil therapy in liver-transplant patients with SARS-CoV-2 infection was associated with an increased risk of a severe course of COVID-19 (RR 3.94, P = 0.003)[39]. Therapies with calcineurin inhibitors or everolimus, instead, have been shown to not be associated with an increased likelihood of adverse outcome. Furthermore, discontinuation of immunosuppressive therapy did not show benefits[39]. On the contrary, Webb *et al*[45] did not find any correlation between the type of immunosuppressant and mortality rate in patient with previous LT and SARS-CoV-2 infection. They highlighted that LT seems to not significantly increase the COVID-19-related mortality rate. These data are supported by the results of a meta-analysis including the main studies performed on LT patients with SARS-CoV-2 infection[46]. In these patients the 30-d mortality was comparable to the mortality rate found in the general population (OR: 0.90, 95%CI: 0.55-1.47). In light of unavailability of univocal data, the European Association for the Study of the Liver suggests to personalize immunosuppressive therapy changes based on patient's medical history, disease severity and the type of ongoing immunosuppressive therapy[47].

CHRONIC HEPATITIS AND COVID-19 CLINICAL OUTCOMES

As mentioned above, if liver cirrhosis is associated with high rates of COVID-19-related mortality, several data indicate that patients with chronic hepatitis do not show an increased risk[15]. However, in addition to the stage of the disease, the different etiology could also affect the COVID-19 outcomes (Figure 1).

Alcohol-related liver disease

During the pandemic, the relationship between alcohol and SARS-CoV-2 infection has been shown to be bidirectional. On one hand, the isolation and socio-economic uncertainties resulting from the COVID-19 pandemic have led to an increase in alcohol consumption[48], already on the rise in the last 20 years [49]. On the other hand, several studies reported that alcohol-related liver disease (ALD) seems to be associated with a poorer prognosis for COVID-19 than the other etiologies[15,25,30,50]. In this regard, Marjot *et al*[25] showed ALD to be an independent risk factor for death from COVID-19 (OR: 1.79; 95%CI: 1.03-3.13). Similar results have been obtained from Mallet *et al*[15] and Kim *et al*[30]. Recently Bailey *et al*[50] confirmed that alcohol use disorders (AUDs) worsen the COVID-19 course and are associated with an increased hospitalization rate and all-cause mortality compared to patients with SARS-CoV-2 infection but without AUDs.

AUDs are already known as a risk factor for ARDS and ARDS-related multiorgan failure[51]. In fact, chronic alcohol consumption has been demonstrated to cause significative alterations in epithelial and endothelial cell function, surfactant synthesis and secretion, lung matrix composition and alveolar-capillary barrier function. Such alterations could increase susceptibility to respiratory pathogens, like SARS-CoV-2, leading to higher ARDS rates and adverse outcomes compared to patients without AUDs. Furthermore, ethanol exposure could stimulate the activity of key inflammatory mediators with a pro-inflammatory response further exacerbated by SARS-CoV-2 infection, resulting in a more severe course of COVID-19[52].

Chronic viral hepatitis

Several studies analyzed the mutual interaction between chronic hepatitis B virus (HBV) and SARS-CoV-2 infection, investigating whether the underlying viral disease could determine a worse prognosis during the COVID-19 course. Numerous data suggest that patients with chronic HBV infection have similar characteristics to HBV-negative patients in prevalence of laboratory abnormalities (changes in cytolysis and cholestasis liver markers), severity of the COVID-19 course and mortality[53-55]. The absence of a significant correlation between COVID-19-related outcomes and chronic viral hepatitis is confirmed by meta-analysis of Sarkar *et al*[56], in which the Authors found no significant impact on overall mortality during SARS-CoV-2 infection. Neither the degree of HBV replicative activity seems to affect the SARS-CoV-2 infection outcomes; inactive carriers or patients with previous infection have ALI and mortality rates comparable to patients with active hepatitis[57,58]. As further demonstration of the absence of correlation between HBV replicative activity and COVID-19-related outcomes, antiviral therapy for HBV is not able to determine a significant impact on mortality, need for admission to the ICU and hospitalization length[59]. Apparently, conflicting with these data, Yang *et al*[60] showed that

the HBeAg-positive chronic HBV hepatitis are associated with a higher rate of hospitalization in ICU and mortality. However, the Authors did not stratify the study cohort in relation to disease stage and the impact of the presence of liver cirrhosis and organ failure on these results is unknown. Finally, the role and safety of immunosuppressive therapies (*e.g.*, corticosteroids, IL-6 pathway inhibitors such as tocilizumab) used in cases of SARS-CoV-2-related ARDS were evaluated for the risk of HBV reactivation in patients with biohumoral signs of previous infection (HBsAg-negative, HBcAb-positive). In these patients, the risk of HBV reactivation following immunosuppressive treatment for COVID-19 appears negligible and not influenced by any antiviral prophylaxis[61].

Little data are available to date on the association between the severity of COVID-19 course and chronic hepatitis C virus (HCV) infection. Some studies report an increase of mortality for patients with chronic HCV infection[62]. However, also in this case, the proportion of patients with liver cirrhosis and the related impact on outcomes is unknown. Butt *et al*[63] showed similar COVID-19-related mortality rates among HCV-positive patients compared to HCV-negatives, despite a higher hospitalization rate. However, in the study population, HCV patients show a higher prevalence of liver cirrhosis than those not with HCV (8.1% *vs* 1.4%, respectively; $P < 0.0001$). Lastly, Cerbu *et al*[64] investigated the differences in outcomes between patients with active HCV infection and those who were under treatment or achieved sustained virological response. They found that patients with active infection showed an overall worse prognosis in terms of hospitalization, severe COVID-19 course, ICU admission and all-cause mortality compared to non-viremic patients. Regarding this, the early treatment with sofosbuvir/velpatasvir combination (used for HCV infection) in patients with COVID-19 has been shown to be effective in speeding up the clearance of SARS-CoV-2 and preventing disease progression [65].

Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is currently the most frequent etiology of liver disease worldwide, affecting approximately 32.5% of the global population[66]. It is closely associated to metabolic comorbidities such as obesity, diabetes mellitus, arterial hypertension and chronic kidney failure[67]. Such comorbidities related to NAFLD have been shown to play a predictive role for adverse outcomes in COVID-19, being associated with higher rates of hospitalization, mechanical ventilation and mortality[19,68]. For these reasons, great attention has been paid to determine whether NAFLD itself could represent an independent prognostic factor in COVID-19. However, studies in this setting are affected by the variability in the definition of NALFD patients, using for this purpose clinical-anamnestic or radiological (by ultrasound or computed tomography) criteria or score [hepatic steatosis index (HSI)] in different ways. Data currently available are not univocal. In one of the very first reports, Ji *et al*[69] showed that, net of comorbidities, NAFLD (diagnosed by ultrasound or by a value > 36 of the sums of HSI and body mass index) was an independent predictor of COVID-19 progression (OR: 6.4; 95%CI: 1.5-31.2). Furthermore, NAFLD was associated with higher prevalence of ALI during hospital stay and a slower viral clearance compared to the control group without NAFLD. Mahamid *et al*[70] later confirmed these data, despite the small cohort size. Conversely, in a recent case control study, NAFLD was not found to be associated with higher in-hospital mortality rates, need for ventilatory support, ICU admission, or overall length of hospital stay[71]. Similar results have been obtained by Marjot *et al*[25] and Kim *et al*[30]. Also, in the study by Vrsaljko *et al*[72], NAFLD is not shown to be independently correlated to a severe course of COVID-19 and to mortality rates in the multivariate analysis, while it appears significantly related to the hospitalization length and the incidence of pulmonary thrombosis.

Moreover, the nomenclature of NALFD recently has been changed to metabolic associated fatty liver disease (MAFLD)[73]. At the same time, the diagnostic criteria have been redefined and the results do not overlap with the previous ones. These new criteria have also been recently applied in the setting of patients with SARS-CoV-2 infection, showing conflicting data in this case as well. In this regard, Vá zquez-Medina *et al*[74] reported that patients with MAFLD, but not those with NAFLD, have higher mortality rates (55.0% *vs* 38.3%; $P = 0.02$) than the control group not MAFLD/not NAFLD, whereas both MAFLD and NAFLD are associated with a higher rate of orotracheal intubation. Gao *et al*[75] confirmed that MAFLD increases by 4 times the risk of a severe course of COVID-19 and the association remains even after adjusting for age, sex, and comorbidities. Surprisingly, some preliminary data would indicate that the correlation between MAFLD and severity of COVID-19 course is more significant in patients under 60[76]. In contrast to the above-mentioned studies, Campos-Murguía *et al*[77] observed that fibrosis rather than MAFLD is associated with a severe course of COVID-19 (increased need for mechanical ventilation, increased incidence of acute kidney injury), and higher mortality. However, in most of these studies enrolled patients were not evaluated for the possible presence of liver cirrhosis. This could represent a significant bias with a potential impact on the results. As expected, the presence of intermediate or advanced liver fibrosis in patients with MAFLD is indeed associated with a higher risk of severe COVID-19[78]. In this regard, advanced fibrosis has been shown to determine a significant increase in mortality risk both in patients diagnosed with MAFLD and in those diagnosed with NAFLD compared to patients without CLD[74] and patients with mild or moderate liver fibrosis[79].

Recently, a large-scale 2-sample Mendelian randomization analysis had been carried out in order to provide possible conclusive data on the association between NAFLD and the COVID-19 course[80]. Although NAFLD is associated with a severe course of SARS-CoV-2 infection in univariate analysis, this association disappears after adjustment for age, sex, and comorbidities. Therefore, the Authors conclude that there is no evidence supporting that NAFLD is a causal risk factor for severe COVID-19. The results favoring this association are probably attributable to the correlation between NAFLD and obesity.

Autoimmune liver disease

The incidence of COVID-19 in patients with autoimmune hepatitis (AIH) is comparable to that of the general population[81,82]. Despite that available data are limited, the main studies agree that patients with AIH do not present significant differences in hospitalization rates, disease severity and mortality during SARS-CoV-2 infection compared to patients with non-AIH CLD and the general population[83, 84]. However, steroid treatment during COVID-19, when not indicated, can cause a more severe course of the disease. While steroids represent a cornerstone in the therapy of SARS-CoV-2-related ARDS[85, 86], particularly when associated to antiviral drugs[87], their use is not recommended in the absence of respiratory failure due to lack of benefit and potential worsening of outcomes[85,86]. In this regard, Efe *et al*[88]recently highlighted that the use of corticosteroids or azathioprine for AIH therapy is associated with a significant increase in the risk of severe form of COVID-19 (OR: 4.73; 95%CI: 1.12-25.89), even after adjusting for demographic characteristics, comorbidities and presence of liver cirrhosis. However, in the absence of conclusive data, any remodulation of immunosuppressive therapy during SARS-CoV-2 infection should be personalized after a careful assessment of the risks and benefits[47].

Role and efficacy of vaccination in patients with CLD and liver cirrhosis

The global availability of anti-SARS-CoV-2 vaccines in the last months of 2020 has resulted in a reduction of hospitalization and mortality rates from COVID-19 despite the succession of different viral variants[89]. Given their vulnerability profile in cases of SARS-CoV-2 infection, such vaccines have been strictly recommended in LT recipients and patients with CLD, with priority to cirrhotic patients and those with HCC or ALD (Figure 1)[90-92]. In this setting, vaccines have found to be generally safe[93-95], although sporadic cases of post-vaccinal ALI are reported, with predominantly hepatocellular and immune-mediated injury due to a probable aberrant response of the immune system after vaccine stimulation[96].

Despite the strong indication, patients with CLD (particularly those with liver cirrhosis) appear underrepresented in phase III trials of anti-SARS-CoV-2 vaccines, both for mRNA and viral vector ones [97-99]. In fact, CLD and in particular the presence of significant liver fibrosis could negatively affect the production of innate immunity proteins and the count and performance of B and T lymphocytes[100]. For this reason, in the last 2 years, a growing number of clinical studies have investigated the efficacy of anti-SARS-CoV-2 vaccines in patients with CLD. Anti-SARS-CoV-2 vaccines are able to determine both a T-cell and a humoral response, stimulating the production of antibodies directed against the Spike protein (seroconversion) and neutralizing antibodies[101,102]. Prospective studies compared the immunological response induced by a full course of mRNA vaccines and/or viral vector vaccines in patients with liver cirrhosis and controls[93-95]. Almost all patients with liver cirrhosis showed production of anti-Spike antibodies and a neutralizing antibody activity, similar to patients without liver cirrhosis[93]. A recent meta-analysis confirmed comparable seroconversion rates between cirrhotic patients and patients with CLD without cirrhosis[100]. Despite comparable seroconversion rates, Iavarone *et al*[95] reported a statistically significant difference in the antibody titer developed by patients with liver cirrhosis after vaccination compared to controls (1751 U/mL *vs* 4523 U/mL; P = 0.012). In particular, CP classes B and C and the presence of HCC would appear to be independently associated with low levels of antibody titer. We hypothesize that this suboptimal vaccine response could potentially indicate partial protection against SARS-CoV-2 infection and a reduction in its duration, particularly in patients with decompensated cirrhosis. Furthermore, with regard to the cellular response induced by the vaccine, the production of interferon-gamma after spike-specific stimulation of T lymphocytes is detectable only in 65.4% of patients with liver cirrhosis, against 100% of controls[94].

Beyond humoral response, little data are available on clinical efficacy of anti-SARS-CoV-2 vaccines in patients with CLD. The most important knowledge on this issue have been acquired from Veterans Outcomes and Costs Associated with Liver Disease cohort[103] and National COVID Cohort Collaborative registers[104]. In the first study, John *et al*[103] compared overall and COVID-19-related mortality 60 d after SARS-CoV-2 infection in cirrhotic patients receiving mRNA vaccine and cirrhotic patients not previously vaccinated. Unequivocally, postvaccination COVID-19 was associated with reduced mortality rates (HR: 0.21; 95%CI: 0.11-0.42) compared to unvaccinated patients with liver cirrhosis, with an overall reduction in the risk of death of approximately 80%. The benefit of vaccination is also statistically significant in patients with decompensated cirrhosis and in those who have not completed the vaccination course[103]. Similar results have been reported also by Ge *et al*[104], whose study is available only in "pre-print" version to date. This study, carried out during SARS-CoV-2 alpha and delta variants predominance, describes a 66% reduction in 30-d mortality in vaccinated cirrhotic patients compared to unvaccinated patients with liver cirrhosis. Furthermore, the administration of the third dose of mRNA vaccine in cirrhotic patients seems to lead to an 80% reduction in cases of SARS-

CoV-2 infection (symptomatic or asymptomatic) and a 100% reduction in the severe forms of COVID-19 and related death compared to the administration of two doses, overcoming the hyporesponsiveness to vaccines in these patients[105]. The impact of the third vaccine dose appears stronger among patients with compensated rather than decompensated cirrhosis.

If patients with liver cirrhosis show overall suboptimal but effective seroconversion rates on protection against risk of death and a severe COVID-19 course, data obtained from LT recipients appear less encouraging. In fact, LT recipients show lower vaccine response[94,100,106]. Seroconversion rates in these patients range from 47.5% and 65%, significantly lower than controls[94,107]. Overall, 28% of patients undergoing LT did neither develop a T-cell nor a humoral response after vaccination[94]. An optimal humoral response is developed in only one third of liver transplant patients[107]. Among the factors associated with vaccine response rate in this setting, studies agree in identifying advanced age, alcoholic etiology of liver disease, and immunosuppressive therapy as independent predictors of reduced antibody response[93,106,108]. Conclusive data on the correlation with the specific immunosuppressive regimen are not available to date. Some studies would indicate that the reduced antibody response is particularly significant for patients treated with mycophenolate mofetil[106,108] or high doses of steroids[106]. Other manuscripts reported instead a negative correlation with calcineurin inhibitors compared to other immunosuppressive drugs[94]. Thuluvath *et al*[107] highlights that the use of ≥ 2 immunosuppression drugs is associated with poor immune response.

Finally, at the moment, few data are available about the influence of CLD etiology on vaccine-induced immune responses. Among the various etiologies, despite the high seroconversion rates, AIH patients show a significantly lower antibody titer than both patients with autoimmune cholestatic liver disease and controls, independently from the presence of liver cirrhosis and the ongoing immunosuppressive therapy[109]. Despite this, the clinical benefit of vaccination appears consistently in AIH patients, showing a significative reduction in hospitalization, severe course and COVID-19-related mortality rates (adjusted OR: 0.20)[110].

CONCLUSION

Patients with CLD are more vulnerable to SARS-CoV-2 infection. In particular, patients with liver cirrhosis show higher hospitalization rates, severe COVID-19 course and mortality than the general population. Mortality rates increase according to stage of cirrhosis. Among etiologies, ALD is the most frequently associated to adverse outcomes. Patients with NAFLD have high mortality rates and severe COVID-19 course in relation to the high burden of comorbidities. Anti-SARS-CoV-2 vaccination is safe and effective in patients with CLD: in particular, patients with liver cirrhosis benefit from a complete vaccination course. Patients who have undergone liver transplant show higher mortality rates and a reduced humoral response to vaccination. In any case, vaccination should be encouraged in all patients with CLD, particularly for those at higher risk due to disease stage and related etiology.

FOOTNOTES

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Country/Territory of origin: Italy

ORCID number: Riccardo Nevola 0000-0003-3320-3878; Antonio Russo 0000-0002-4224-5343; Raffaele Marfella 0000-0003-3960-9270; Luigi Elio Adinolfi 0000-0001-8453-4912; Ferdinando Carlo Sasso 0000-0002-9142-7848; Aldo Marrone 0000-0001-7329-4159; Luca Rinaldi 0000-0002-6541-3821.

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Outcomes of COVID-19 among patients with liver disease

Isidora Vujčić

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Isidora Vujčić, Institute of Epidemiology, Faculty of Medicine, University of Belgrade, Belgrade 11000, Belgrade, Serbia

Corresponding author: Isidora Vujčić, MD, PhD, Associate Professor, Institute of Epidemiology, Faculty of Medicine, University of Belgrade, Visegradska 26a, Belgrade 11000, Belgrade, Serbia. isidora.vujcic@med.bg.ac.rs

Abstract

Coronavirus disease 2019 (COVID-19) is primarily a respiratory disease with multi-organ involvement, including impaired liver function. It has been noticed that a significant proportion of COVID-19 patients have liver dysfunction, especially those with a more severe disease course. The coronavirus causes direct damage to the liver using the angiotensin-converting enzyme 2, a cell-surface receptor for cellular entry, that is expressed in the liver. According to previous research, liver enzyme abnormalities were observed in a considerable proportion of COVID-19 patients, and elevated liver transaminases were found in about 20% of these patients, alkaline phosphatase in 6.1%, and gamma-glutamyl transferase in 21.1%. COVID-19 might trigger a deterioration of liver function in patients with pre-existing chronic liver diseases (CLDs) and also in those without previous liver disorders. The majority of COVID-19 patients who develop liver injury are men, the elderly, and those with a higher body mass index. Compared to the general population, COVID-19 is associated with significant morbidity and mortality in patients with liver disease (cirrhosis and liver transplantation recipients). However, some studies indicate that CLDs have a lesser role in determining patient progression towards higher disease severity.

Key Words: Liver disease; COVID-19; Mortality; Prognosis; Liver function

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Core Tip: Drastic lifestyle changes during the coronavirus disease 2019 (COVID-19) pandemic have led to an increase in the incidence of liver disease. Liver damage in COVID-19 infection occurs during disease progression in patients with or without previous liver disorders and represents a risk factor for developing severe illness and death. The prognosis of COVID-19 infection depends predominantly on the patients' characteristics, present comorbidities, severity of clinical symptoms, laboratory parameters, and imaging features. It is important to examine prognostic factors in COVID-19 disease patients with liver disease because it may improve the outcome.

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INTRODUCTION

The coronavirus disease (COVID-19) originated in late 2019 in China and spread with alarming rapidity across the globe[1]. The illness is caused by a novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and during the pandemic, more than 600 million cases and more than 6 million deaths were reported worldwide[2]. COVID-19 clinical manifestations vary, and the disease's wide clinical spectrum ranges from mild, self-limiting pulmonary tract infection to progressive severe pneumonia with high mortality rates[3]. Drastic lifestyle changes during the COVID-19 pandemic have led to an increase in the incidence of nonalcoholic fatty liver disease (NAFLD), decompensated cirrhosis, acute alcoholic hepatitis, viral hepatitis, and mortality from liver diseases[4]. Liver dysfunction in COVID-19 patients is a risk factor for severe illness and death[5], and significantly higher morbidity and mortality rates were observed among patients with liver disease and COVID-19, compared to the general population[6]. About 2%-11% of COVID-19 patients had already been diagnosed with chronic liver disease (CLD)[7]. However, liver dysfunction includes a variety of etiologies and heterogeneous groups of patients[8]. In addition, COVID-19 can induce liver injury, especially in those patients with severe forms of the disease[7,9].

GLOBAL BURDEN OF LIVER DISEASES

Globally, two million deaths are attributed to liver diseases, including 1 million due to cirrhosis and 1 million due to viral hepatitis and hepatocellular carcinoma (HCC)[10]. Over the past two decades, the prevalence of CLD has been increasing[11]. CLD includes NAFLD, alcohol-related liver disease (ALD), and chronic viral hepatitis B and C[12], and it can progress to fibrosis, cirrhosis, and HCC[13]. NAFLD, or the recently defined metabolic-associated fatty liver disease (MAFLD), are the most common CLDs, which affect about a quarter of the world's adult population[14]. The global prevalence of MFALD/NAFLD has been rapidly increasing in tandem with the rise in diabetes and obesity prevalence, both of which have been associated with increased mortality in COVID-19[15,16]. Hepatitis B and C are still a major cause of liver disease burden globally, especially in low-income countries in Asia and sub-Saharan Africa, despite the availability of effective preventive measures and treatment[10,17].

LIVER INJURY IN CORONAVIRUS INFECTED PATIENTS

Although coronavirus can cause the worst damage to the lungs, it can also influence the digestive, cardiac, and endocrine systems[18]. Multifactorial causes of liver damage during COVID-19 infection include direct virus cytopathogenic effect, abnormal immune response associated with the cytokine storm, vascular changes due to coagulopathy, hepatic ischemia/hypoxia reperfusion injury, and drug-induced liver injury[19,20]. The coronavirus causes direct liver injury using the angiotensin converting enzyme 2 receptor for cellular entry, which is expressed mainly in the cholangiocytes and less frequently in the hepatocytes[21]. Certain hepatotoxic medications, such as antibiotics (macrolides, quinolones), antivirals (ribavirin), steroids, and other drugs used to treat patients with COVID-19, are connected with drug-induced liver injury and were found in 10.9% of COVID-19 patients[22-24]. However, in COVID-19 patients, liver damage is primarily secondary to ischemic, hypercoagulable, and hyperinflammatory states, which are independent predictors of death rather than liver injury per se [21]. A cytokine storm and a massive acute-phase response are defined by the acute overproduction and uncontrolled release of the proinflammatory cytokines, tumor necrosis factor (TNF), interleukin 1 (IL-1),

and IL-6 paralleled by excessive secretion of C-reactive protein (CRP) and ferritin[25]. Coagulation dysfunction indicates a poor outcome in critically ill COVID-19 patients with hepatic injury, including a significant role of neutrophils and monocytes in amplifying blood clotting[20]. Hepatic apoptosis and elevated liver enzymes are caused by ischemia and reperfusion injury[20].

PROGNOSTIC FACTORS IN COVID-19 PATIENTS WITH LIVER DISEASE

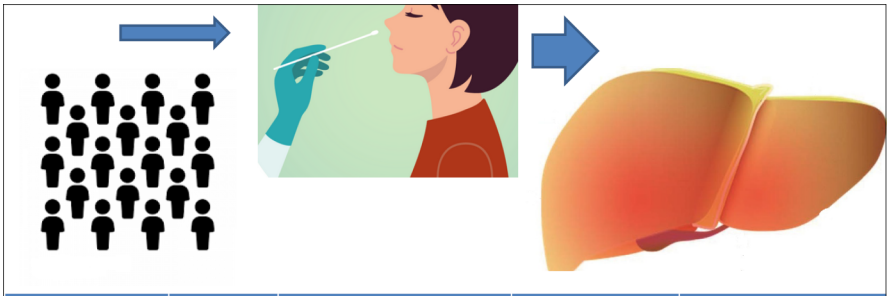
Advanced age and being male are well-established risk factors for severe COVID-19 outcomes[26]. Various medical underlying conditions, such as cardiovascular disease, lung disease, cancer, diabetes, and obesity have also been associated with increased risk[27-29]. However, the prognostic factors in COVID-19 patients with previous liver diseases are not well-defined[9]. In a multicenter cohort study conducted in the United States, comorbidities such as diabetes, hypertension, chronic obstructive pulmonary disease, current smoking and increasing age in patients with ALD, liver cirrhosis decompensation, and HCC predicted a higher mortality when infected with COVID-19[11]. In another study from the United States conducted among CLD patients, it was reported that older age and pre-existing comorbidities were associated with severe COVID-19[30]. Shen *et al*[31] found that COVID-19 patients with liver injury had a significantly poorer prognosis than patients without liver dysfunction, and that male sex and elevated CRP were independent prognostic factors in these patients[31]. Preliminary results of a systematic review and meta-analysis involving 88 studies and 6653207 cases of COVID-19 in Europe showed that liver disease was associated with hospital admission and mortality, after adjustment for age and sex[32]. Liver dysfunction during COVID-19 has been associated with increased disease severity, prolonged hospital stays, ventilatory support and mortality[33].

Liver injury, laboratory findings, and prognosis

The prognosis of the COVID-19 infection depends primarily on the patients' characteristics, present comorbidities, severity of clinical symptoms, laboratory parameters, and imaging features[34] (Figure 1). Liver injury occurs in patients with or without pre-existing liver disorders[24]. The incidence of liver injury manifesting as abnormal levels of liver enzymes ranges from 14.8% to 53.0%[18]. The degree of liver injury is generally mild, and those with digestive symptoms were more likely to present hepatocellular injury[3,35,36]. COVID-19 patients who develop liver injury are more likely to be men, older, and have a higher body mass index (BMI)[37]. Liver enzyme abnormalities are frequent in patients with COVID-19 infection, and they are associated with disease severity[18,38]. The most frequently reported mild to moderate elevations were in aspartate aminotransferase (ALT), alanine aminotransferase (AST), and total bilirubin (tBIL) levels[35], but abnormal gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), and albumin levels have been found in patients with COVID-19 as well[18,22,39]. Liver damage in COVID-19 is usually temporary, and therefore, the enzyme levels of most patients usually return to normal after recovery[18,40]. The systematic review and meta-analysis that included 36 studies and 20724 patients found a 46.9% prevalence of at least one abnormal liver function test, and elevated levels of ALT, AST, and tBIL were independent predictors of COVID-19 severity and in-hospital mortality[41]. A meta-analysis of observational studies revealed that acute liver injury and elevated liver enzymes in COVID-19 patients were significantly associated with disease severity[42]. A study conducted in Hong Kong reported that, ALT/AST elevation at two times the upper normal limit and acute liver injury in patients with COVID-19, were independently associated with poor prognosis, after controlling for diabetes mellitus, hypertension, and albumin level[43]. A systematic review that included 30 articles observed a significantly higher mortality in patients with impaired liver function than in patients with normal function[36]. Wagner *et al*[44] reported that hypoalbuminemia and abnormalities in liver function tests may be prognostic factors for higher COVID-19 severity. Although, there remains controversy in the scientific literature over whether or not liver enzyme abnormalities are associated with worse clinical outcomes, their alteration probably reflects the systemic involvement of the virus and the potential appearance of severe liver complications[45]. However, patients with severe COVID-19 may show a higher risk of post-COVID cholangiopathy, and liver tests in these patients continue to show abnormal results[46].

COVID-19 IN PATIENTS WITH PRE-EXISTING LIVER DISEASE

A number of studies have investigated the impact of CLD on the outcome of COVID-19[8]. COVID-19 patients with CLD account for less than 1% of the reported cases[47]. CLD includes different etiologies and can manifest from mild asymptomatic disease to severe decompensated cirrhosis, so it could be challenging to generalize results from different studies and countries[11,17]. In China, the main cirrhosis etiology was chronic hepatitis B virus (HBV)[48]. Patients with viral hepatitis, ALD, NFALD, liver cirrhosis, and HCC had a higher risk of developing severe COVID-19 and up to a 10-fold higher mortality rate compared to those without any reported comorbidity[49]. Data collected from 13 Asian



Demographic	Habits	Clinical	Laboratory	Chronic liver disease
Advanced age	Alcohol use	Severity of liver disease	Altered liver enzymes	Cirrhosis
Male gender	Smoking	Gastrointestinal symptoms		MAFLD/NAFLD
Higher BMI		Comorbidities		Alcohol-related liver disease
				HBV and HCV

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Figure 1 Prognostic factors in COVID-19 patients with liver disease. BMI: Body mass index; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NAFLD: Nonalcoholic fatty liver disease; MAFLD: Metabolic-associated fatty liver disease.

countries identified that COVID-19 infection induced significant liver damage in CLD patients, and these patients had a higher risk of getting acute liver injury, hepatic decompensation, or acute-on-chronic liver failure (ACLF)[50]. A Danish prospective, population-based cohort study reported that patients with CLD, particularly those with cirrhosis, were at a major risk of severe COVID-19 outcomes and higher mortality[51]. A Swedish nationwide matched cohort study showed that patients with CLD had a higher risk of hospitalization for COVID-19 compared to the general population[52]. However, there was no evidence that these patients were at a higher risk of developing a severe COVID-19 disease course[52]. A study conducted in China reported that COVID-19 patients with CLD showed a prolonged length of stay, slight liver injuries, and higher mortality rates compared to COVID-19 patients without CLD, and that the neutrophil-to-lymphocyte ratio was an indicator of adverse clinical outcomes in this population[53]. A meta-analysis that included fifty studies revealed that pre-existing liver diseases or acute liver injury associated with severe COVID-19 infection were key factors in the prediction of mortality[54]. According to a study conducted in Massachusetts, United States, CLD in patients with COVID-19 was independently associated with higher rates of intensive care unit (ICU) admission, and a need for mechanical ventilation after controlling for comorbidities[12]. Krishnan *et al* [30] found that CLD patients with elevated AST and tBIL levels had a significantly higher risk fora more severe COVID-19 disease course and also reported that ALD was the most important factor associated with the need for mechanical ventilation. A systematic review including 40 studies, mainly from China, reported that CLD was significantly associated with COVID-19 severity and mortality[55]. The risk of getting more severe COVID-19 was 2.44 times higher among patients with CLD compared to those without CLD, and the presence of NAFLD was the most strongly associated with higher COVID-19 severity, followed by MAFLD and cirrhosis. In addition, COVID-19 patients with viral hepatitis were not at higher risk of getting a severe form of COVID-19[55]. After COVID-19 infection, approximately 20% of CLD patients develop progressive cholestasis, particularly patients with NAFLD/non-alcoholic steatohepatitis and metabolic risk factors[56].

COVID-19 and viral hepatitis

There is still insufficient evidence for an association between previous hepatitis B and C infection and COVID-19 outcome, and several studies indicated that these patients were not at increased risk for severe COVID-19[57-59]. Most studies that have examined the influence of HBV on COVID-19 prognosis have been conducted in China due to the high prevalence of HBV in the country[60]. Yu *et al* [61] reported higher in-hospital mortality, more severe disease, and liver function abnormalities in COVID-19 patients infected with HBV compared to COVID-19 patients without HBV. However, the presence of COVID-19 infection or treatment with tocilizumab or corticosteroids could reactivate hepatitis B infection[49,62]. A study conducted in the United States reported that chronic hepatitis C in COVID-19 patients was associated with in-hospital mortality regardless of baseline comorbidities, admission values of laboratory tests, or liver damage induced by COVID-19[63]. The Korean nationwide population-based cohort study reported that after adjusting for age, sex, cirrhosis, and comorbidities, HBV infection itself appears not to influence the prognosis of COVID-19 patients[64].

COVID-19 and NAFLD

The prevalence of NAFLD, or the recently renamed MAFLD, in COVID-19 patients is 31%, which is higher than the prevalence in the general population[65]. Patients with NAFLD had a higher risk of COVID-19 progression, a higher likelihood of liver dysfunction, and a longer viral shedding time than the patients without NAFLD[66]. Mahamid *et al*[16] found an independent association between the COVID-19 severity and NAFLD irrespective of the metabolic syndrome, indicating that NAFLD had a significant impact even in the absence of obesity and/or metabolic syndrome. A systematic review and meta-analysis showed that the proportion of patients with MAFLD and NAFLD ranged from 28% to 50% and from 6% to 38%, respectively, and found that the presence of MAFLD and NAFLD was associated with worse clinical outcomes for COVID-19[67]. Although several studies also showed significant associations between MAFLD and NAFLD and severe COVID-19 outcomes[13,68], there is still no strong evidence that the presence of MAFLD affects its prognosis[65].

COVID-19 and ALD

The COVID-19 pandemic probably had the biggest effect on patients with ALD due to substantially increased alcohol consumption provoked by adverse economic effects, disruptions in work and education, and social isolation[69]. Patients with alcohol use disorders are more likely to develop acute respiratory distress syndrome and have additional comorbidities such as metabolic syndrome, chronic kidney disease, and smoking, all of which are independent predictors of COVID-19 severity[69]. Several studies have identified that ALD is independently associated with COVID-19 mortality after adjustment for important cofactors such as liver disease severity[11,70].

COVID-19 and cirrhosis

COVID-19 patients with cirrhosis are at a greater risk of adverse outcomes than the background population[26,71,72]. Cirrhotic patients have significantly higher all-cause mortality in COVID-19 infection than non-cirrhotic patients, and mortality is probably higher in those with more advanced cirrhosis[8]. A significantly higher COVID-19 related morbidity and mortality had been observed in patients with decompensated cirrhosis compared to those with compensated cirrhosis[6]. Studies conducted in the United States and Europe reported that patients with CLD who had acquired COVID-19 had high rates of hospitalization and mortality[71,73,74]. Marjot *et al*[70] reported that patients with cirrhosis had a higher risk of dying from COVID-19 and that mortality was especially high among patients with more advanced cirrhosis and those with ALD. Hashemi *et al*[12] demonstrated that the presence of cirrhosis was independently associated with COVID-19-related mortality. Similar results were obtained from the United States study, reporting that the presence of decompensated cirrhosis was an independent predictor of mortality in COVID-19 patients[30]. Jeon *et al*[75] reported that the COVID-19 infection in patients with cirrhosis was more likely to cause severe complications in comparison with the cirrhotic patients not infected with COVID-19. Satapathy *et al*[76] found that the development of ACLF was the most important predictor of higher in-hospital mortality in COVID-19 patients with cirrhosis. However, it is still unknown whether the presence of liver disease influences the natural history of COVID-19 infection in cirrhotic patients[21].

COVID-19 and hepatocellular carcinoma

The presence of HCC in patients with CLD and COVID-19 infection was associated with a poor prognosis, including a higher risk of all-cause and COVID-19-related mortality[11]. Most HCC patients have concomitant cirrhosis, and that could potentially increase their risk for severe COVID-19[77]. An international, multicenter, retrospective, cross-sectional study, including two hundred fifty patients from 38 centers, reported that 18.4% of patients with HCC died within the first 30 d from the onset of COVID-19 symptoms, and that the mortality rate in that period was 20.25% in patients with HCC history and 12.96% in those with de novo HCC[78]. COVID-19 in HCC patients tends to be more severe and leads to exacerbation of the liver disease[79]. HCC patients infected with COVID-19 are at a higher risk of complications, ICU admission, and death than the patients without cancer[80].

COVID-19 and autoimmune liver disease

Autoimmune liver disease (AILD) includes primary sclerosing cholangitis, primary biliary cholangitis (PBC), autoimmune hepatitis (AIH), and overlapping syndromes referring to the coexistence of two autoimmune diseases[81]. COVID-19 outcomes in patients with AILD were investigated in international registry studies and retrospective case studies[82]. Combined data from three multinational registries showed that despite the use of immunosuppressive treatment, AIH patients did not seem to have a higher risk of lethal outcomes with COVID-19 compared to patients without liver disease and those with other forms of liver disease[83]. A retrospective study from 34 centers in Europe and the Americas indicated that patients with AIH did not have an increased risk for poor prognosis with COVID-19 than other causes of CLD and that cirrhosis was the most important predictor for high COVID-19 severity in this group of patients[84]. Zecher *et al*[85] indicated that patients with AILD were not at elevated risk for COVID-19. A Spanish nationwide study reported that cumulative incidences of hospitalization and COVID-19 related mortality were greater in patients with PBC than in the general Spanish population,

although the results were not adjusted for other comorbidities[86].

Severity and mortality of COVID-19 among CLD patients

Although the presence of COVID-19 infection in CLD patients is associated with a poor prognosis, including severity and mortality, these results should be interpreted with caution and need to be evaluated in large future studies. Such findings could be explained by overlapping risk factors, therapeutic effort limitations, different etiologies, and the disease spectrum of CLD, which ranges from mild asymptomatic disease to severe decompensated cirrhosis. Cirrhosis severity and older age are the most important predictors of mortality[21]. A French national retrospective cohort study found no increased COVID-19 severity in patients with CLD, alcohol use disorders, cirrhosis, or primary liver cancer, indicating that the COVID-19 outcome in these patients may be more associated with therapeutic effort such as mechanical ventilation and less with liver disease progression or ethanol toxicity[87]. This group of patients was at an elevated risk for mortality from COVID-19 within 30 d after admission but was less likely to need mechanical ventilation[87] in comparison with patients with mild liver diseases, compensated cirrhosis, chronic viral hepatitis, non-viral, non-alcoholic causes of CLD, acquired immunodeficiency syndrome, and liver transplantation, who were not at a higher risk of dying from COVID-19 but were more likely to receive mechanical ventilation[87]. As mentioned earlier, the Swedish nationwide cohort study also did not find an increased risk of getting severe COVID-19 in CLD patients, although they had an increased risk of hospitalization than the background population [52]. The pooled analysis of six studies found that CLD was not related to an elevated risk of a more severe COVID-19 disease course or mortality[88]. Similar results were reported from a nationwide Korean cohort study indicating that LC was not an independent predictor of severe complications, including mortality, in COVID-19 patients and depended on age, hypertension, cancer, chronic obstructive pulmonary disease, and a higher Charlson comorbidity index[75]. After adjustment for age, sex, BMI, cardiac disease, hypertension, diabetes, and respiratory disorders, CLD, and NAFLD were independently associated with ICU admission and the need for mechanical ventilation, but not death [12].

CONCLUSION

Due to the era of the COVID-19 pandemic and the large number of patients with liver disease, it is very important to study the impact of liver damage on the prognosis of patients with COVID-19 and the predictors that may affect the outcome. Identifying predictors of mortality could allow for risk stratification of patients and help improve healthcare delivery[11]. Therefore, it is necessary to improve the understanding of host genetics, behavior, and pre-existing comorbidities and adequately follow-up liver disease patients[17]. Patients with CLD, especially those with cirrhosis or advanced liver damage, should be prioritized for COVID-19 vaccination[89].

FOOTNOTES

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ORCID number: Isidora Vujčić 0000-0003-3442-7400.

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MINIREVIEWS

Bone loss in chronic liver diseases: Could healthy liver be a requirement for good bone health?

Jelena Jadzic, Danijela Djonic

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Jelena Jadzic, Danijela Djonic, Center of Bone Biology, Faculty of Medicine, University of Belgrade, Belgrade 11000, Serbia

Corresponding author: Danijela Djonic, MD, PhD, Professor, Center of Bone Biology, Faculty of Medicine, University of Belgrade, Dr. Subotica 4/2, Belgrade 11000, Serbia.
ddjonic@yahoo.com

Abstract

Given that the liver is involved in many metabolic mechanisms, it is not surprising that chronic liver disease (CLD) could have numerous complications. Secondary osteoporosis and increased bone fragility are frequently overlooked complications in CLD patients. Previous studies implied that up to one-third of these individuals meet diagnostic criteria for osteopenia or osteoporosis. Recent publications indicated that CLD-induced bone fragility depends on the etiology, duration, and stage of liver disease. Therefore, the increased fracture risk in CLD patients puts a severe socioeconomic burden on the health system and urgently requires more effective prevention, diagnosis, and treatment measures. The pathogenesis of CLD-induced bone loss is multifactorial and still insufficiently understood, especially considering the relative impact of increased bone resorption and reduced bone formation in these individuals. It is essential to note that inconsistent findings regarding bone mineral density measurement were previously reported in these individuals. Bone mineral density is widely used as the "golden standard" in the clinical assessment of bone fragility although it is not adequate to predict individual fracture risk. Therefore, microscale bone alterations (bone microstructure, mechanical properties, and cellular indices) were analyzed in CLD individuals. These studies further support the thesis that bone strength could be compromised in CLD individuals, implying that an individualized approach to fracture risk assessment and subsequent therapy is necessary for CLD patients. However, more well-designed studies are required to solve the bone fragility puzzle in CLD patients.

Key Words: Chronic liver disease; Fracture risk; Hepatic osteodystrophy; Osteoporosis; Bone strength

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Core Tip: Secondary osteoporosis and increased bone fragility are frequently overlooked complications in patients with chronic liver disease (CLD). Recent publications agree that CLD-induced bone fragility depends on the etiology, duration, and stage of liver disease, but certain ambiguities are still present. Importantly, etiopathogenetic mechanisms leading to CLD-induced bone loss are still insufficiently clarified. Given that available clinical tools for fracture risk assessment are not entirely reliable, evaluating small-length structural bone properties could improve understanding of the multifactorial nature of bone fragility in CLD patients, which could set a base for the development of more effective preventive and therapeutic strategies.

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INTRODUCTION

The importance of a wide range of liver functions in the human body becomes the most visible in chronic liver disease (CLD). The most commonly known CLD complications are portal hypertension, hepatic encephalopathy, ascites, hepatorenal syndrome, variceal bleeding, and hepatocellular carcinoma [1,2]. However, CLD is also associated with changes in the skeleton, previously known as hepatic osteodystrophy[3,4]. Among CLD patients, substantial heterogeneity of skeletal changes was noted depending on the etiology, duration, and stage of the liver disorder[5,6]. Namely, osteoporosis was initially described as a complication of primary biliary cholangitis and primary biliary cirrhosis (cholestatic liver diseases)[7], while skeletal changes were later described in other (non-cholestatic) hepatic disorders as well[8,9]. It has been reported that approximately every second patient with viral hepatitis, hemochromatosis, and Wilson's disease has osteoporosis or osteopenia[10-12], while up to 55% of patients with alcoholic liver cirrhosis have osteoporotic bone changes[3,13,14]. Interestingly, bone alterations in nonalcoholic fatty liver disease or nonalcoholic steatohepatitis have recently drawn researchers' attention, revealing that up to one-third of these individuals could develop bone alterations [15,16].

Consequently, CLD individuals are at substantial risk for non-traumatic bone fractures[17-19], with a prevalence between 7% and 35%[20]. Recent data suggest that fracture incidence is two to three times higher in end-stage CLD patients compared to healthy controls[19,21], while others reported an eight-fold increase in the risk of bone fractures in these patients[22]. Regarding fracture localization, data suggest that vertebral fractures are most common in patients with end-stage CLD[19,23-26], given that more than one-third of these individuals experienced at least one vertebral fracture during their lifetime [8,23,27]. Moreover, CLD contributes to the age-associated increase in the risk of femoral fracture and subsequently its life-threatening complications[22]. It is important to emphasize that end-stage CLD patients are experiencing fragility fractures at a significantly younger age than most osteoporotic patients[22], considering that the cumulative fracture risk in CLD patients younger than 45 years corresponds to the risk of healthy controls over 75 years of age[22]. It is important to emphasize that CLD likely changes the sex distribution of fracture risk in the aged population, considering that CLD is more frequent in male patients[28], while osteoporosis and osteoporosis-related bone fractures are more likely to develop in older women[29].

Despite the significant number of studies that have assessed various characteristics of bone deterioration in CLD individuals, many unknowns should be elucidated to understand this topic entirely.

OSTEODENSITOMETRY FINDINGS IN CLD PATIENTS

Most studies dealing with bone changes in CLD patients used dual-energy X-ray absorptiometry as the most valuable tool in the clinical assessment of fracture risk[30]. Interestingly, opposite results were yielded. Namely, dual-energy X-ray absorptiometry assessment revealed significantly lower bone mineral density (BMD) in patients with viral, autoimmune, and primary biliary cirrhosis[31-33]. At the same time, other authors failed to show a significant BMD decrease in CLD of the same etiology[34,35]. Multiple studies showed reduced dual-energy X-ray absorptiometry-obtained BMD values, suggesting osteopenia or osteoporotic changes of the lumbar spine and femoral neck in patients with alcohol-induced CLDs[36-38], while other research teams failed to show these bone alterations in individuals prone to chronic alcohol abuse[17,39,40]. Given that the primary source of these contradictory data could be in the study design (cross-sectional study design), selection criteria, and the number of participants included in the study, future well-designed prospective studies are required to fully

understand BMD alterations in CLD patients.

BONE TURNOVER BIOMARKERS IN PATIENTS WITH CLD

As a non-invasive and cost-effective tool for indirect assessment of bone remodeling dynamics, bone turnover biomarkers (BTMs) are a complementary method in the clinical management and follow-up of the treatment effects in patients with osteoporosis and osteoporosis-related bone fragility[41]. Automated or manual immunoassays using blood or urine samples are utilized to measure a specific combination of these protein or protein-derivative biomarkers[42], which are considered indicative of the dynamic relationship between osteoblast activity (bone formation markers) and osteoclast activity (bone resorption markers)[41,43]. The most frequently investigated bone formation markers are osteocalcin, bone alkaline phosphatase, and N-propeptide of type I collagen[41]. On the other side, commonly interpreted bone resorption markers are C-terminal and N-terminal telopeptides of type I collagen, deoxypyridinoline, and tartrate-resistant acid phosphatase isoform 5b[41] (Figure 1).

The interpretation of BTM levels has been of clinical utility in age-related osteoporosis[43], while its role in the clinical management of CLD-induced bone loss is still modest. Some data suggest that serum levels of osteocalcin and bone alkaline phosphatase are decreased in individuals with CLD[25,36,44], while others failed to show significant differences between individuals with CLD and the control group [45,46]. Moreover, contradictory data regarding the level of β-CTX and deoxypyridinoline were noted in CLD patients[36,45,47,48]. It is important to note that liver dysfunction could affect serum concentrations of BTMs, which reveals excessive bone matrix degradation, indicating that its assessment allows only limited conclusions in CLD individuals[10,49]. Multiple limitations of BTM assessment are among the reasons why CLD-induced bone changes are recognized and treated after a patient experiences non-traumatic fracture[10], suggesting that further investigation is required to elucidate the role of BTMs in developing novel, adequate preventive and treatment strategies.

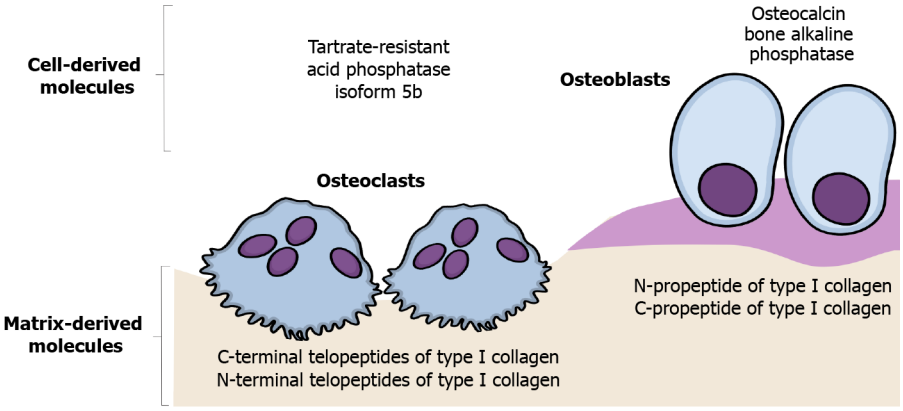
ASSESSMENTS OF MICROSCALE BONE PROPERTIES IN CLD INDIVIDUALS

The World Health Organization recommended BMD as the primary parameter for the diagnosis of osteopenia and osteoporosis and for clinical fracture risk assessment[50]. However, considering that the occurrence of fragility fractures primarily requires the action of several bone strength determinants and their mutual interaction, it is evident that increased bone fragility could not be solely explained by BMD decrease[51,52]. In other words, low BMD should only be considered an applicable and non-invasive clinical surrogate marker of bone fragility[52,53]. Namely, it has been known that only up to one-third of non-traumatic fractures are attributable to low BMD values, indicating that many individuals with bone fractures have BMD in the referent range[54].

Moreover, various bone properties are recognized as important determinants affecting bone strength (ability to resist fracture)[55]. Thus, current studies suggested that multiscale analysis of various bone properties (with respect to the hierarchical structure of the bone, Figure 2) could contribute to a better understanding of increased bone fragility in elderly individuals with chronic comorbidities, including a variety of CLDs[56]. The importance of assessing these bone properties is highlighted by the fact that some pharmaceutical agents were proven to improve bone strength and reduce fracture risk without increasing BMD[57,58], indicating the potential for developing new and effective treatment strategies [52].

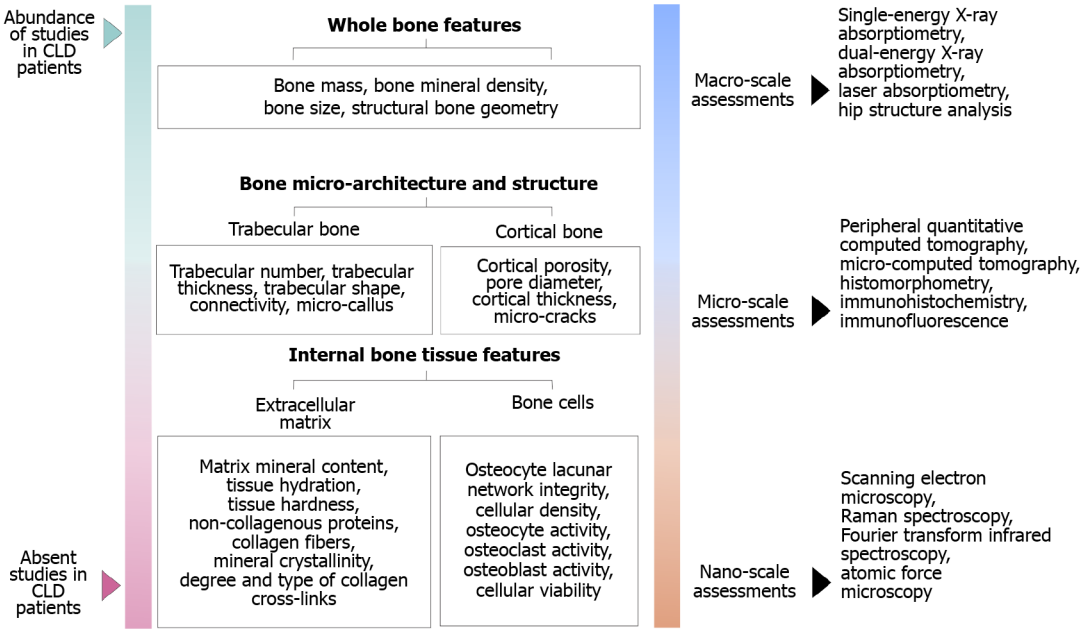
Initially, histomorphometry studies using optic microscopy assessment of iliac bone biopsies showed deteriorated trabecular bone architecture in CLD patients[59,60]. In addition, some novel clinical studies confirmed these results on the tibia and radius of CLD patients, using a newer methodology called peripheral quantitative computed tomography[33,61,62]. Since osteoporosis is not uniform throughout the skeleton[63] it was crucial to assess CLD-induced microstructural decline in lumbar vertebrae and proximal femora[38,64]. Similarly to previous findings, our research group used microcomputed tomography with an isotropic resolution of 10 μm to observe impaired microarchitectural integrity of lumbar vertebrae and proximal femora collected from CLD individuals[9,38,64]. On the trace of altered trabecular and cortical microarchitecture, we demonstrated reduced mechanical bone competence in these individuals[38,65], indicating that altered bone matrix content could be involved in CLD-induced bone fragility.

Future state-of-the-art studies should focus on a precise nanoscale morphostructural estimate of the inorganic (mineral) and organic component of the bone extracellular matrix (collagen fibers) to elucidate its role in increased bone fragility among CLD individuals (Figure 2). Finally, the long-term benefit of small-length bone studies could develop a specific diagnostic algorithm that will help to reliably predict bone strength based on the information available in the clinical context of each patient.



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Figure 1 Schematic representation of the most frequently analyzed bone turnover markers. The emphasis is placed on the difference between bone turnover markers released by catabolic osteoclast activity (bone resorption markers) and anabolic osteoblast activity (bone formation markers).



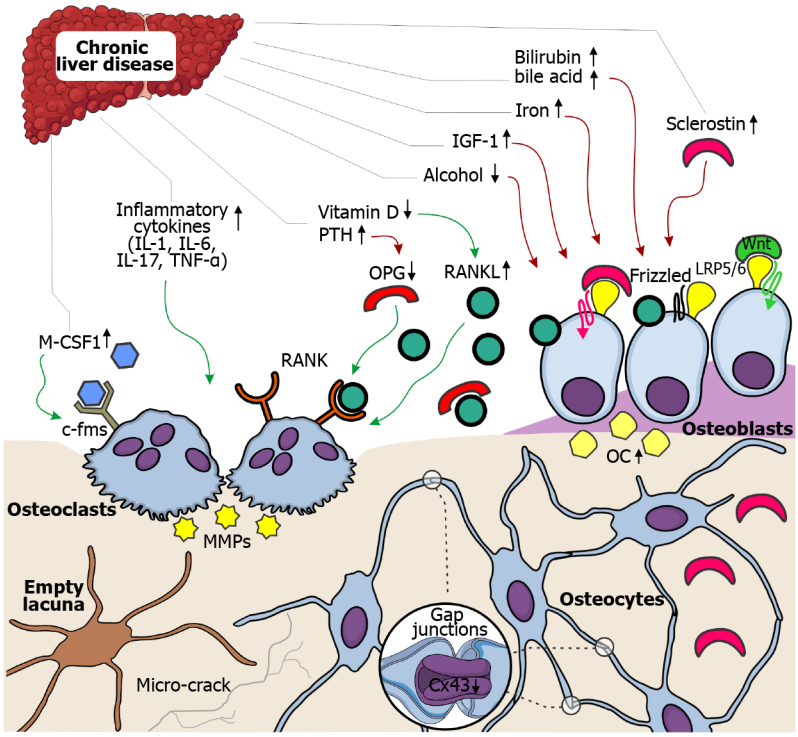
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Figure 2 Multiscale approach in the assessment of bone strength determinants. The importance of the various bone properties that contributes to increased bone fragility, and up-to-date methodologies are used to assess these bone strength determinants. The emphasis is placed on the difference between factors that were previously assessed and those factors that require further investigation in patients with chronic liver disease. CLD: Chronic liver disease.

THE MOLECULAR MECHANISMS INVOLVED IN ETIOPATHOGENESIS OF CLD-INDUCED BONE LOSS

Bone loss in CLD patients is commonly described as a consequence of bone remodeling disturbance[8], but the particular contribution of increased bone resorption and decreased bone formation still needs to be thoroughly explained. Nowadays, a common understanding is that the etiopathogenetic mechanisms of bone loss are dependable on the etiology of liver disease[3,8]. Previous data revealed that osteoblast dysfunction and decreased bone formation play a central role in the etiopathogenesis of bone loss in patients with cholestatic liver disease, Wilson's disease, and hemochromatosis[7,12,48,66]. Conversely, viral CLD displays a more dominant effect on increased osteoclast activity, inducing high-turnover osteoporosis[21,32,67].

On a molecular level, low-turnover osteoporosis in CLD patients is commonly associated with toxic effects of biliary stasis and copper/iron accumulation on differentiation, maturation, and proliferation of osteoblasts (Figure 3)[68-70]. Also, previous studies suggested that osteoblast dysfunction in patients with cholestatic forms of CLD could be mediated by insulin growth factor-1 or oncofetal fibronectin[66,70,71], while direct toxic effects of alcohol on osteoblastic function contribute to bone loss among patients within alcohol-induced CLD[72,73]. During the process of bone formation, osteoblasts become



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Figure 3 Schematic representation of possible pathophysiological mechanisms leading to bone loss in chronic liver disease patients. The role of multiple factors leading to bone loss and osteoporosis in individuals with chronic liver disease places an emphasis on the difference between factors that cause osteoblast dysfunction (reduced bone formation) and factors that stimulate osteoclast activity (increased bone resorption). Green arrows indicate an activating effect, while red arrows indicate a deactivating effect. c-fms: Colony-stimulating factor-1 receptor; Cx43: Connexin 43; IGF-1: Insulin-like growth factor 1; IL: Interleukin; LRP5/6: Low-density lipoprotein receptor-related protein 5/6; M-CSF1: Macrophage colony-stimulating factor 1; MMPs: Matrix metalloproteinases; OC: Osteocalcin; OPG: Osteoprotegerin; PTH: Parathyroid hormone; RANK: Receptor activator for nuclear factor kappa B; RANKL: Receptor activator for nuclear factor kappa B ligand; TNF: Tumor necrosis factor.

embedded within the bone matrix, continuing to function as bone remodeling orchestrators or osteocytes[74]. Osteocytes form a global network throughout the bone tissue by intercellular channels (gap junctions), most frequently formed by connexin 43[75]. Reduction in osteocytic expression levels of connexin 43 and minor disruptions in the osteocyte lacunar network was noted in CLD individuals (Figure 3), suggesting that the mechanosensing potential and molecular transduction might be defective in those patients with CLD[65,76]. In addition, increased bone expression levels of sclerostin (an osteocyte-derived negative regulator of bone formation) were noted in CLD individuals[65,76], which was in accordance with previous clinical studies[77,78]. These data indicate that treatment targeting sclerostin may be an interesting strategy to fight osteoporosis in CLD patients[10]. Still, possible therapeutical utilities in CLD patients are yet to be thoroughly investigated in the years ahead.

Previous studies revealed that bone loss in CLD individuals could be explained by a strong link between systemic hyperproduction of inflammatory mediators and increased bone resorption (Figure 3)[21,32,67]. Most commonly, it is understood that tumor necrosis factor-α, interleukin (IL)-1, IL-6, IL-7, IL-11, IL-13, IL-15, and IL-17, produced by immune cells, could directly activate osteoclast precursors or display an indirect effect by osteoblasts[8,10,72]. Namely, increased secretion of receptor activator for nuclear factor kappa B ligand (RANKL), the disturbed ratio between RANKL and osteoprotegerin, matrix metalloproteinases activity, and cathepsin K are described as contributing factors in CLD-induced bone loss *via* increased bone resorption (Figure 3)[10,79-81]. The recent recommendation for therapy targeting RANKL advocates the importance of the RANK-RANKL-osteoprotegerin system in bone loss among CLD patients[20,82]. In addition, increased circulating macrophage colony-stimulating factor 1 in CLD patients could promote bone resorption due to its role in priming a larger number of monocytes to form osteoclasts in these patients[6].

Lastly, low vitamin D levels, unbalanced diet (low calcium and protein intake), malabsorption, disruption in the homeostasis of the intestinal microbiome, coupled with a variety of hormonal and metabolic disruptions (such as increased levels of parathyroid hormone, hypogonadism, and hypercorticism) were identified as factors that contribute to bone loss in CLD individuals[20,72,83]. Based on these data, new nutritional support guidelines were recently introduced by the European Association for the Study of the Liver[20,84]. However, given that bone changes in CLD patients are undoubtedly present, it is vital to further investigate more direct etiopathogenetic mechanisms involved in the relationship between liver and bone disorders.

CONCLUSION

Bone alterations are a common complication in patients with CLD, especially in those with liver cirrhosis. Over the previous period, numerous studies have contributed to understanding bone fragility in CLD patients. However, numerous ambiguities are still present due to the modest reliability of clinical diagnostic methods, which could lead clinicians to doubt whether or when it is necessary to start treating CLD-induced skeletal alterations. Thus, evaluating small-length structural bone properties could improve understanding of the multifactorial nature of bone fragility in CLD patients. All these data could set a base for developing a patient-specific diagnostic algorithm that will reliably predict bone strength based on the information available in a clinical context. Additionally, specific clinical guidelines for preventing, diagnosing, and treating skeletal disorders in patients with CLD need to be established in the near future.

FOOTNOTES

Author contributions: Jadzic J and Djonic D contributed to conceptualization; Jadzic J contributed to data acquisition, writing the original draft, and data visualization; Djonic D contributed to reviewing and editing and supervision; All authors approved the submitted version of the manuscript.

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Country/Territory of origin: Serbia

ORCID number: Jelena Jadzic 0000-0002-3124-1400; Danijela Djonic 0000-0003-1043-1077.

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MINIREVIEWS

Liver involvement in patients with COVID-19 infection: A comprehensive overview of diagnostic imaging features

Davide Ippolito, Cesare Maino, Federica Vernuccio, Roberto Cannella, Riccardo Inchingolo, Michele Dezio, Riccardo Faletti, Pietro Andrea Bonaffini, Marco Gatti, Sandro Sironi

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Davide Ippolito, Pietro Andrea Bonaffini, Sandro Sironi, Milano Bicocca School of Medicine and Surgery, Milano 20126, Italy

Davide Ippolito, Cesare Maino, Fondazione IRCCS San Gerardo dei Tintori, Monza 20900, Italy

Federica Vernuccio, Institute of Radiology (DIMED), University Hospital of Padova, Padova 35128, Italy

Roberto Cannella, Section of Radiology-Department of Biomedicine, Neuroscience and Advanced Diagnostics (BiND), University of Palermo, Palermo 90127, Italy

Roberto Cannella, Department of Health Promotion, Mother and Child Care, Internal Medicine and Medical Specialties (PROMISE), University of Palermo, Palermo 90127, Italy

Riccardo Inchingolo, Michele Dezio, Division of Interventional Radiology, Department of Radiology, Madonna delle Grazie Hospital, Matera 75100, Italy

Riccardo Faletti, Department of Surgical Sciences, University of Turin, Turin 10126, Italy

Pietro Andrea Bonaffini, Sandro Sironi, Department of Diagnostic Radiology, Papa Giovanni XXIII Hospital, Bergamo 24127, Italy

Marco Gatti, Department of Diagnostic Radiology, University of Turin, Turin 10126, Italy

Corresponding author: Davide Ippolito, MD, Associate Professor, Doctor, Milano Bicocca School of Medicine and Surgery, Piazza dell'Ateneo Nuovo, 1, Milano 20126, Italy.
davide.atena@tiscali.it

Abstract

During the first wave of the pandemic, coronavirus disease 2019 (COVID-19) infection has been considered mainly as a pulmonary infection. However, different clinical and radiological manifestations were observed over time, including involvement of abdominal organs. Nowadays, the liver is considered one of the main affected abdominal organs. Hepatic involvement may be caused by either a direct damage by the virus or an indirect damage related to COVID-19 induced thrombosis or to the use of different drugs. After clinical assessment, radiology plays a key role in the evaluation of liver involvement. Ultrasonography (US), computed tomography (CT) and magnetic resonance imaging (MRI) may be used to evaluate liver involvement. US is widely available and it is

considered the first-line technique to assess liver involvement in COVID-19 infection, in particular liver steatosis and portal-vein thrombosis. CT and MRI are used as second- and third-line techniques, respectively, considering their higher sensitivity and specificity compared to US for assessment of both parenchyma and vascularization. This review aims to the spectrum of COVID-19 liver involvement and the most common imaging features of COVID-19 liver damage.

Key Words: Liver; Fatty liver; Hepatomegaly; Hepatic infarction; Liver diseases; Liver failure; Biliary tract diseases; COVID-19; SARS-CoV-2; Infection; X-Ray computed tomography; Magnetic resonance imaging; Ultrasonography; Adults; Pediatrics

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Core Tip: Coronavirus disease 2019 (COVID-19) infection has an impact not only on lung involvement but also in other systems, in particular the gastrointestinal one, with a special focus on the liver. Hepatocytes express the receptor of angiotensin-converting enzyme which is the main door of the entrance of severe acute respiratory syndrome coronavirus 2. Consequently, different mechanisms can lead to different hepatic scenarios, such as hepatomegaly, steatosis, steatohepatitis, and drug-induced liver injury. As for lung involvement, the infection can lead to hepatic vascular involvement, especially portal vein thrombosis. Finally, it has been demonstrated a possible biliary involvement in COVID-19 patients.

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INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, named coronavirus disease 2019 (COVID-19), represents an epoch-making global healthcare crisis, with 603711760 confirmed cases and 6484136 deaths caused to date worldwide[1].

Although the lung represents the most affected organ, COVID-19 may present as a multiorgan disease. Clinical manifestations may vary from flu-like symptoms, such as fever, dry cough, myalgia, and fatigue, often coupled with hypo-/anosmia and ageusia[2-4], to more severe conditions with dyspnea and respiratory impairment requiring admission to the intensive care unit (ICU) and advanced respiratory assistance[5]. A severe course of the disease has been reported in 5%-22 % of COVID-19 patients[3,5].

In this scenario, we focused our attention on hepatic manifestations of COVID-19 infection. Hepatic involvement in patients with COVID-19 infection is not negligible. Liver damage can occur in different ways, ranging from hepatomegaly, acute hepatitis, steatosis and steatohepatitis, portal vein thrombosis (PVT) and liver infarction, biliary and gallbladder involvement, up to drug-induced liver injury (DILI), with chronic liver disease that needs further long-term studies to be understood (Figure 1).

In this review, we aim to describe the spectrum of COVID-19 liver involvement and the most common imaging features of COVID-19 liver damage with a descriptive correlation to the underlying pathogenesis.

IMAGING TECHNIQUES

The standard radiological approach for liver assessment (*i.e.* anatomy, focal liver lesions, or diffuse diseases) has been widely described and does acknowledge the use of ultrasound (US), computed tomography (CT), and magnetic resonance imaging (MRI). Technical advances in liver imaging have also been conducted over the last decades, with lots of research on quantitative and functional assessments in different liver pathologies[6].

US

Brightness-mode (B-mode) transabdominal US generally represents the first-line approach in patients with suspected liver disease[7]. US is widely available, non-invasive, low cost, safe, ionizing radiation-free[6] and can be performed at the bedside, particularly in ICU or isolated patients. Anatomical and

vascular imaging and lesion detection are feasible, although limited by the field of view and are dependent on operator experience[7].

During the last decades, different US developments have been introduced, including elastography, contrast-enhanced US (CEUS), and novel doppler techniques[6-9].

Moreover, an advanced multiparametric US approach for the evaluation of the liver could be an option, particularly for long-term follow-up of COVID-19 patients. The multiparametric US includes elastography, share wave dispersion, and attenuation imaging. The evaluation by the 2D-shear wave elastography technique allows quantification of the increase in liver stiffness related to the evolution toward fibrosis, but it can be altered in the inflammatory early stages (*e.g.*, steatohepatitis)[10]. Shear-wave dispersion is a measure of liver viscosity that is changed during inflammatory processes in the liver. Finally, hepatic attenuation imaging is a useful tool for quantifying steatosis.

Elastography enables the assessment of liver fibrosis[6]. Usually, a quantitative assessment of liver stiffness is obtained by applying an external force by means either of a US-induced focused impulse (point shear wave elastography) or a mechanically induced impulse (transient elastography)[7]. More recent developments of US elastography include a volumetric assessment of liver stiffness and its real-time variations. Clinical use of US elastography is mainly limited by cutoff values for fibrosis staging that vary across US systems from different vendors[6].

CEUS is accepted as a second-line imaging modality for the characterization of focal liver lesions after inconclusive baseline US, and its cost-effectiveness is higher compared to CT or MRI[6,7,11]. CEUS interpretation is similar to CT and MRI, relying on the similar post-contrast phases (arterial, portal-venous, delayed), vascular architecture, and phase-specific enhancement of the lesion compared with the adjacent liver parenchyma[7]. CEUS is useful for lesion detection and characterization in several clinical settings, without the use of ionizing radiations and with higher temporal resolution compared to CT or MRI[6]. It can be useful in non-oncologic, non-cirrhotic patients[6,9], for the assessment of incidental focal lesions, in cirrhotic patients, allowing characterization of contrast enhancement patterns of hepatocellular carcinoma (HCC) with good sensitivity and specificity[6], and in oncologic patients, providing higher sensitivity compared to the standard US for liver metastases detection and indeterminate CT or MRI lesions characterization[12]. CEUS can also be used to guide, in real-time, both focal lesions procedures[7,8] and locoregional ablative therapies, as well as for treatment response assessment[13,14]. According to the latest guidelines[14], US contrast agents can be safely administered in various applications, with minimal risks to patients. The reported rate of anaphylactoid-type reactions is extremely rare (0.014%).

Among novel third-generation doppler developments, US manufacturers have introduced techniques such as superb microvascular imaging (SMI) that has improved the sensitivity and accuracy of Doppler US in the assessment of hepatic vascular anatomy[9] and the detection of liver tumors vascularity with a safe, inexpensive, and readily available modality[6]. SMI is based on an adaptive algorithm that separates low flow signals from overlaying tissue motion artifacts; thereby SMI allows visualization of microscopic vessels (either native or within lesions), with no need for contrast agents injection[9].

CT

CT represents the mainstay technique for liver imaging, with the majority of acquisitions performed with multiphase acquisition protocols, and standardized assessment based on size and density measurements. Due to its wide availability, CT is generally preferred to MRI in daily clinical practice, despite its overall lower sensitivity. Moreover, reproducibility and high temporal and spatial resolutions allow its employment in both standard and emergency settings[6,15-17].

Contrast-enhanced CT allows the characterization of liver lesions deemed indeterminate on US in non-oncologic and non-cirrhotic patients. In cirrhosis, while the US remains the standard technique for follow-up, contrast-enhanced CT and MRI are the currently recommended techniques for the characterization of US-detected nodules, diagnosis and post-treatment follow-up of HCC[6,18]. In oncologic patients, CT is generally used for staging and follow-up. Accurate timing of image acquisition during the various dynamic phases is critical to enable a correct determination of liver lesion characteristics and enhancement features[11]. In these scenarios, CT acquired during the portal-venous phase is the most common performed study in oncologic patients[6], but its main limitation is the detection and characterization of small hypoattenuating lesions and lesion detection in the background of liver steatosis[6,19]. On the other hand, multiphase scans (arterial, portal-venous, and delayed phases) are generally used in cirrhotic patients, for focal liver lesions characterization, and in trauma patients[6,8].

More advanced and emerging techniques include perfusion CT, dual-energy CT (DECT) and photon-counting detector CT (PCD-CT)[6,17].

DECT is based on CT data acquisition by using X-rays generated at two different energy spectra; therefore, allowing for superior materials discrimination and characterization. Images are obtained either with dual-source, ultra-fast kV switching, or sandwich detector[6,16]. Then, DECT data post-processing generates several types of images: monochromatic image reconstructions, useful to improve iodine contrast visualization; attenuation maps of different elements according to their atomic number, including iodine, calcium, and water[6]. Moreover, the possibility of generating virtual unenhanced (VUE) images may help reduce radiation dose exposure. DECT improves the delineation of hypo- and hypervascular liver lesions by increasing the lesion to parenchyma contrast. Given the possibility of

material decomposition, DECT can also allow distinguishing contrast from calcifications, and noninvasively quantifying fat, iron, and other moieties, compensating for the high cost and examination time length of MRI and invasiveness of biopsy[6,16,17]. However, DECT is affected by some shortcomings, including technical limitations (limited field-of-view, reduced spectral separation depending on vendor or scanner) and software challenges (lack of enough research comparing vendors and scanners' variability on VUE and iodine attenuation values)[16].

PCD-CT is the most recent promising technique but nowadays is still mainly limited to preclinical or small *in vivo* studies in volunteers. More clinical and validation research is therefore needed over a longer time[6].

MRI

MRI is fundamental in the workup of patients with liver disease[6,17,20] and has been addressed as the preferred imaging modality for the characterization of equivocal focal lesions detected by other imaging modalities[17]. Along with appropriate clinical information, MRI can also allow a definitive diagnosis, avoiding in most cases invasive procedures such as biopsy[21].

A properly dedicated MRI liver protocol requires it to be short, comprehensive, standardized, and reproducible[6,17,21]. Pre-contrast MRI, given its higher contrast resolution compared to CT, provides information about tissue and lesion composition (*i.e.* solid or liquid; iron, fat, glycogen, blood products) and lesions cellularity, either neoplastic or inflammatory[6,11,17,22]. Diffusion-weighted imaging (DWI) has been reported to improve the detection and characterization of focal liver lesions, also allowing differentiation of cysts from solid masses. Moreover, by combining hyperintensity on high b-value DWI (hypercellular lesions) with dynamic multiphasic studies, improvement in lesions detection and characterization (in particular for tumors < 2 cm) can be achieved[11].

Contrast-enhanced MRI represents a relevant component of any liver MRI protocol. It provides reliable information about focal lesions characterization, vascular and biliary anatomy, and more recently organ function[17,20,21]. Both gadolinium-based extracellular (ECA) and hepatobiliary (HBA) contrast agents can be used for multiphase imaging[6,17,21]. Morphologic and vascular-related information are obtained with ECA and HBA through the dynamic study[21].

Moreover, HBA provides the ability to acquire images in the hepatobiliary phase (HBP), offering information about hepatocytes uptake and excretion in the biliary system[6,11,20,21]. Therefore, HBP may provide functional information[21]. Indeed, lesions, or abnormalities without hepatocytes or with non-functioning hepatocytes, appear as hypointense compared to the surrounding liver parenchyma [20]. Among reported HBA advantages, it is worth mentioning higher lesion conspicuity with increased sensitivity in lesion detection, and improved lesion characterization with increased ability in the differential diagnosis.

One of the most used advanced MRI techniques useful to detect and characterize focal or diffuse liver disease is DWI. Highly cellular tissues or those with cellular swelling exhibit lower diffusion coefficients, and these aspects can be useful for the evaluation of liver diseases[22].

The evaluation of the biliary tree can be easily made using highly weighted T2 sequences in different planes. In this setting, magnetic resonance cholangiopancreatography (MRCP) is nowadays considered the reference standard for noninvasive biliary evaluation. Thanks to the improvement of MRI techniques, it is now possible to acquire 3D images that can be reformatted in every plane of space by post-processing techniques[23].

LIVER DISEASE INVOLVEMENT

COVID-19 liver injury is defined as any liver involvement that occurs during the course of COVID-19, whether there is a known history of liver disease or not[24]. The presence of liver damage from a laboratory point of view is very common: an increase in liver enzymes is described in around 40% of patients[24], it is greater with severe COVID-19 and at the same time a predictor of adverse events[25,26].

SARS-CoV-2 primarily infects respiratory epithelial cells *via* angiotensin-converting enzyme 2 (ACE2). ACE2 is also expressed at high levels in the endothelium layer of tiny blood arteries, cholangiocytes and in hepatocytes. Furthermore, the SARS-CoV-2 virus may use the gut-liver route *via* the hepatic reticular system to reach the liver[25]. Finally, other organ systems and drugs have a significant influence on the liver. As a result, the causative mechanisms of liver damage in COVID-19 infection are many, including direct cytotoxicity caused by active SARS-CoV-2 replication, immune-mediated liver injury, vascular impairment caused by coagulopathy, endothelium, or cardiac congestion, hypoxic changes caused by respiratory failure, DILI, and exacerbation of the underlying chronic liver disease[25] (Figure 1 and Table 1).

Hepatomegaly and steatosis

COVID-19 causes twice as much liver damage at the cellular level. First, hepatocellular damage occurs, resulting in mild steatosis, lobular and portal inflammation, and areas of apoptosis and necrosis. This

Table 1 Summary of the most common findings in liver involvement due to coronavirus disease 2019 infection

Liver involvement	Ultrasound	Computed tomography	Magnetic resonance
Hepatomegaly	Qualitative criteria: The right lobe extends inferiorly over the lower pole of the right kidney; Rounding of the hepatic inferior border Quantitative criterion: Length of the right liver lobe > 16.5 cm		
Steatosis	Hyperechoic liver in comparison to the spleen or neighboring kidney; Absence of the normal echogenic walls of the portal and hepatic veins; Poor visualization of deep portions of the liver	Unenhanced: Relative hypoattenuation: liver attenuation more than 10 HU less than that of spleen; Absolute low attenuation: liver attenuation lower than 40 HU; C+: Does not add information	IP/OOP imaging: Signal drop out on OOP; T2W: Isointense; T1: Isointense; T1 C+: Does not add significant information
Acute hepatitis	Hepatomegaly; Reduced echogenicity; Steatosis; Peri-portal edema; Reduced Doppler signal in the hepatic artery; Thickening of the gallbladder wall	Hepatomegaly; Homogeneous/heterogeneous hypoattenuation (steatosis); Peri-portal edema; Thickening of the gallbladder wall; Periportal/hepatoduodenal enlarged nodes	IP/OOP imaging: Steatosis can be present; T2W: Diffuse mild increase in signal; Increased signal around the portal system (periportal edema); T1 C+: Periportal enhancement; Thickening of the gallbladder wall; Hilar enlarged nodes
DILI	Nonspecific findings: Hepatomegaly, steatosis, and peri-portal edema can be present		
Portal vein thrombosis	Absent or reduced flow in the portal vein on Color Doppler; Presence of heterogeneous material (focal or diffuse) in the portal vein lumen	Unenhanced: Higher attenuation into the portal vein lumen; Dilation of the portal trunk; C+: Hypoattenuating material into the lumen; Enhancement of vein walls	T2W: Iso- to hyperintense clot according to the phase (acute or subacute); T1: Hyperintense clot; T1 C+: Hypointense material into the lumen; Enhancement of vein walls
Biliary involvement	Focal or diffuse bile duct dilatation (with/without intrahepatic or extrahepatic stones)	Unenhanced: Does not add information; C+: Focal or diffuse bile duct dilatation (with/without intrahepatic or extrahepatic stones); Heterogeneous enhancement of parenchyma; Peri-portal edema	MRCP: Focal or diffuse bile duct dilatation with/without intrahepatic or extrahepatic stones; Stone(s) in the biliary lumen (hypointense); Multifocal biliary strictures alternated with dilated tracts (beaded appearance); T2W: Hypointense stone; Increased T2 signal around the portal system (periportal edema); T1: Hyperintense stone; T1 C+: Heterogeneous enhancement of peri-biliary parenchyma
Acute cholecystitis	Gallbladder wall thickening (> 3 mm); Pericholecystic fluid; Gallbladder distension; Possible sludge	Unenhanced: Gallbladder distension; Possible sludge (hyperattenuating); Pericholecystic fluid; C+: Gallbladder wall thickening (> 3 mm); Inhomogeneous gallbladder wall; Mural or mucosal hyperenhancement	MRCP: May show an impacted stone in the gallbladder neck or cystic duct; T2W: Gallbladder wall thickening (> 3 mm); Inhomogeneous gallbladder wall due to edema; T1: Sludge (hyperattenuating); T1 C+: Inhomogeneous gallbladder wall; Mural or mucosal hyperenhancement

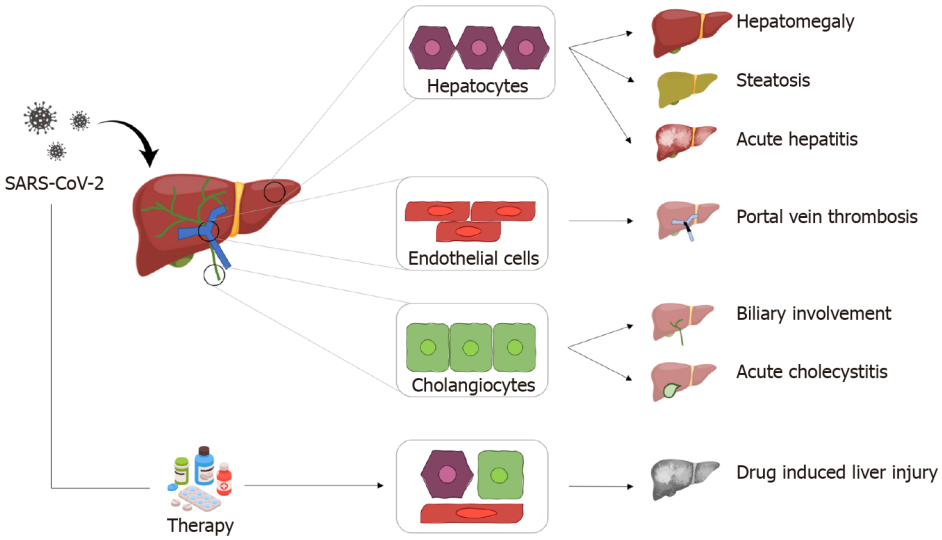
HU: Hounsfield unit; IP: In-phase; OOP: Out-of-phase; C+: Contrast-enhanced; DILI: Drug-induced liver injury; MRCP: Magnetic resonance cholangiopancreatography.

type of damage raises aspartate aminotransferase (AST) and alanine transaminase (ALT) levels. Later on, the damage is direct to cholangiocytes, with bile duct damage and increase in gamma-glutamyl transferase (GGT) and bilirubin[27].

When there is a clinical suspicion of liver involvement in COVID-19, bedside US is the first imaging technique used in the diagnostic workup. A quick and targeted bedside US may be critical in referring selected patients to second-level imaging techniques, to reduce unnecessary exams and diagnostic delays. US can detect morphological or structural changes in the liver: the most encountered findings in COVID-19 patients are hepatomegaly[28,29] and steatosis[29,30].

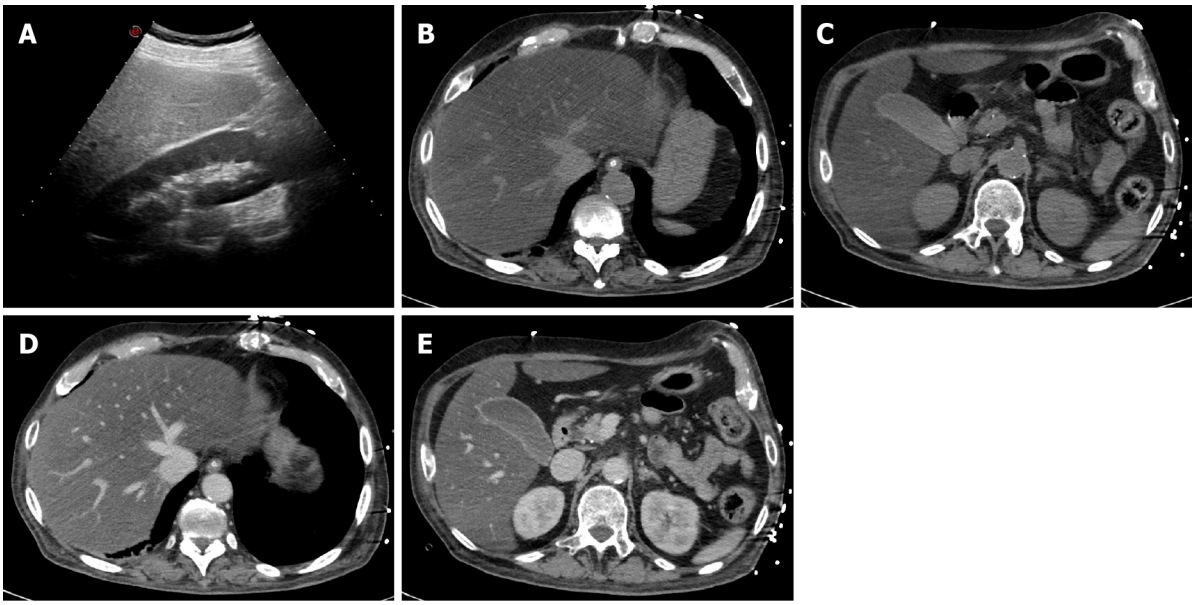
According to Abdelmohsen *et al*[28], the most common morphological change in the liver in critically ill COVID-19 patients is hepatomegaly (about 55% of patients), which is also consistent with autopsies in COVID-19 patients[29-31]. Spogis *et al*[29] found hepatomegaly associated with gallbladder wall thickening and decreased echogenicity (*i.e.* signs of acute hepatitis) in 33% of COVID-19 patients with elevated liver cytolysis indices (> 10-fold). Hepatomegaly is usually identified subjectively during imaging: the "qualitative criteria" include the inferior extension of the right lobe to the lower pole of the right kidney and the rounding of the hepatic inferior border. Otherwise, the quantitative criteria are based on the length of the right liver lobe, with a cutoff of 16.5 cm[31].

On the other side, the most common liver structural change associated with COVID-19 is the presence of hepatic steatosis[28,29] (Figure 2). Coagulation activation can produce hepatic steatosis, and this could be a unique mechanism that leads to both thrombosis and steatosis that are common findings in COVID-19 patients[32]. US B-mode is the most used technique for diagnosing and classifying hepatic steatosis[33], particularly in the moderate or severely affected liver. It has an overall sensitivity and specificity of 85% and 93%, respectively[33]. However, the detection of a moderate degree of steatosis remains poor, with about 60% sensitivity[34]. B-mode US imaging is mostly used to analyze the liver



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Figure 1 Graphical summary of the most common hepatic pathological findings in coronavirus disease 2019. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.



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Figure 2 A 45-year-old woman with coronavirus disease 2019 infection underwent abdominal ultrasonography due to elevated liver enzymes. A: As shown on ultrasonography, the liver is hyperechoic in comparison to the renal parenchyma, suggesting marked steatosis; B-E: The patient underwent contrast-enhanced computed tomography; B and C: On unenhanced images, the liver is diffusely hypoattenuating while its main vessels (*i.e.* portal vein and hepatic veins) appear brighter than the parenchyma due to a marked hepatic fatty infiltration; D and E: On post-contrast images, the liver is homogeneous without any focal lesion; C and E: The gallbladder is filled by sludge, with normal wall thickness. Finally, the combination of clinical and radiological findings allowed the final diagnosis of steatohepatitis.

qualitatively, searching for characteristic markers of steatosis (*i.e.* hyperechoic liver in comparison to the spleen or neighboring kidney)[29]. US findings can be confirmed on abdominal CT. According to Lei *et al*[35], the most common CT abnormalities in COVID-19 patients include diffuse hypoattenuation of the liver (26%) – more common in severe patients (59%) – and the CT-quantified liver/spleen attenuation that can predict prognosis in COVID-19 patients[35,36]. Furthermore, because the liver is partially included in every chest CT, liver data from chest CT scans performed in many COVID-19 patients can be easily retrieved.

Using a multiparametric US approach, Radzina *et al*[30] evaluated 90 patients affected with COVID-19 in the previous 3-9 mo demonstrating how liver elasticity, viscosity, and steatosis are altered after COVID-19 and that these alterations well correlate with liver enzyme abnormalities, even better than CT or MRI findings.

Other consequences of chronic liver disease, such as nonalcoholic fatty liver disease (NAFLD), must be considered in addition to COVID-19-induced liver damage[37]. Obesity and other components of metabolic syndrome have been linked to COVID-19 severity. The impact of NAFLD in COVID-19 patients is controversial in the literature[37]. In a meta-analysis of 1851 patients, Singh *et al*[38] found that, while there was an increase in the course severity of COVID-19 with a 2.60 odds ratio (OR), the adjusted OR (aOR) for mortality risk was 1.01; however, these data should be considered with caution due to the significant heterogeneity among the included studies. Interestingly, Ghoneim *et al*[39] analyzed 8885 patients with common comorbidities known to be linked with COVID-19 and found out that the cumulative incidence of disease was higher if metabolic syndrome was the primary diagnosis (OR 7.0). COVID-19 patients who were African Americans (aOR 7.45), hypertensive (aOR 2.53), obese (aOR 2.20), diabetic (aOR 1.41), hyperlipidemic (aOR 1.70) or had non-alcoholic steatohepatitis (NASH) (aOR 4.93) had higher aORs. These findings demonstrated that, among all concomitant metabolic disorders, NASH had the strongest connection with COVID-19. In support of these data, Roca-Ferná ndez *et al*[40] analyzed 41791 people who underwent MRI for assessment of liver fat, liver fibro-inflammatory disease, and liver iron with proton density fat fraction calculation before February 2020 and found that people with fatty liver (> 10%) had a higher likelihood of testing positive (OR 1.35), and people with obesity and fatty liver had a 5.14 times higher risk of hospitalization. Obese people who did not have a fatty liver can have an increased risk (OR 1.75). According to the findings, obese people with fatty liver disease are at a higher risk of COVID-19 infection and hospitalization.

To summarize, COVID-19 patients have a remarkable risk of liver damage, with the main morphologic and structural changes being hepatomegaly and steatosis, which have a significant impact on patients' prognosis and can be easily studied with US (Table 1). Epidemiologically, people with NAFLD/NASH appear to be at a higher risk of severe COVID-19 infection. However, it is unclear how much of this rise is due to hepatic steatosis or the presence of overlapping risk factors and comorbidities.

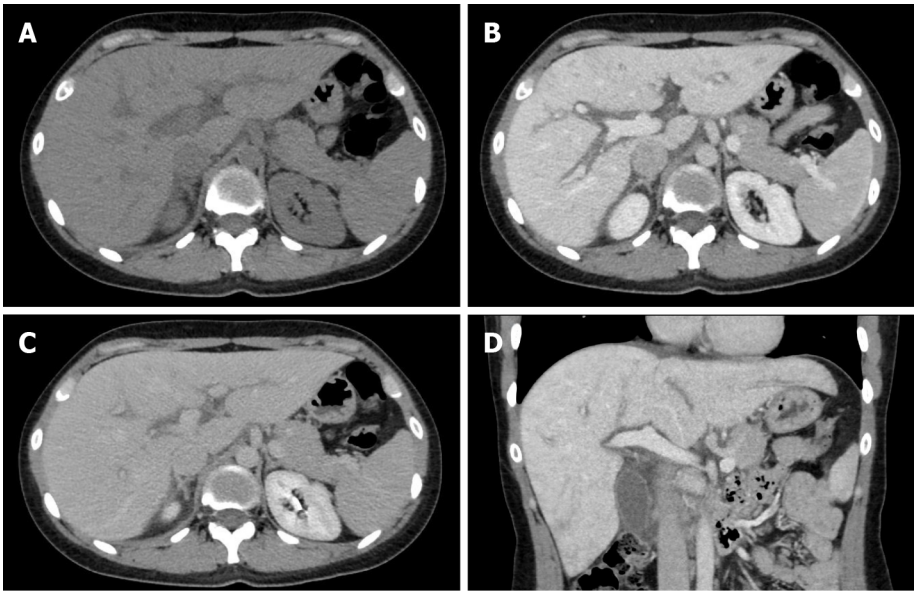
Acute hepatitis in COVID-19

Liver test abnormalities are frequently encountered in COVID-19 patients at admission, and their increase is associated with the severity of the disease[41,42]. In the majority of cases, COVID-19-induced hepatitis occurs as benign new transient hepatitis with gradual onset, elevated AST and ALT levels, and lack of any radiological changes[29,42,43]. Occasionally, COVID-19-induced hepatitis may occur in otherwise asymptomatic patients as the sole manifestation of COVID-19 infection[44]. Liver damage in COVID-19-induced hepatitis may be the result of viral infection of hepatocytes or cholangiocytes, hypercoagulability with both microangiopathy and local thrombus formation, immune-mediated damage, systemic inflammation, or hypoxic hepatitis due to the respiratory disease[45-49]. Regarding the first mechanism, the virus enters the hepatocytes, and then viral replication results in rupture of cells, generating elevated serum liver enzymes[48,50]. Thrombotic complications in COVID-19 patients are likely to occur due to a pro-coagulant effect or a progressive endothelial thrombo-inflammatory syndrome[49]. Interestingly, in a systematic review of pathology studies, hemodynamic compromise and thromboembolic disease in the liver were demonstrated in 48.3% and 39.4%, respectively, while liver microthrombi were not identified[48].

Radiological hallmarks of acute hepatitis in COVID-19 patients are encountered in approximately 8% of patients with mild-to-moderated liver test abnormalities, and the occurrence increases in patients with severe elevation of liver enzymes. Radiological hallmarks of COVID-19-induced hepatitis – particularly in the most severe cases – include thickening of the gallbladder wall, hepatomegaly, reduced echogenicity of the liver on the US or homogeneous/heterogeneous liver hypoattenuation on CT, and reduced Doppler signal in the hepatic artery[29,35,51]. Liver hypoattenuation is significantly more common in the most severe cases, with the decrease of the liver to spleen CT attenuation ratio being significantly correlated with the severity of pulmonary lesions and the overall COVID-19 severity[35] (Figure 3). A pathology-radiology correlation study demonstrated that the histological features in patients with sonographic changes included macrophage activation, centroacinar necrosis, granulocytic and histiocytic infiltrate, endothelial damage, and severe cholestasis[29,52]. Among these, macrophage activation was particularly interesting as it may represent the histopathologic correlate of a hyperinflammatory syndrome[29]. In a case of COVID-19-induced hepatitis being the sole symptom and without any other cause for liver damage, pathology demonstrated periportal and interstitial inflammation with predominantly lymphocytes, rare plasma cells, and neutrophils, hepatocyte rosette formation, apoptotic bodies, centrilobular congestion, and mildly increased portal and pericellular fibrosis[52] (Table 1).

DILI

DILI is a liver dysfunction caused by drugs used as a treatment for COVID-19 disease. Therapeutic choices for COVID-19 have rapidly expanded and changed over time with the increased understanding of the virus and the disease[53]. The various therapeutic treatments used over time included antiviral drugs, antibiotics, antimalarials, immunomodulator agents, antipyretic agents, adjunctive treatments, and several investigational treatments including convalescent plasma administration from COVID-19 recovered patients[54]. Different studies reported that liver injury in patients with COVID-19 infection



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Figure 3 A 33-year-old woman with coronavirus disease 2019 infection underwent contrast-enhanced computed tomography due to a suspicion of portal vein thrombosis. A: On unenhanced phase liver is enlarged, homogeneous and without any focal lesion; B: On the portal venous phase the liver enhancement is slight inhomogeneous associated with peri-portal edema, a typical finding of acute hepatitis; C and D: These aspects can be clearly observed also on the coronal reconstruction acquired on the portal venous phase (D) and the delayed phase (C).

could be a direct consequence of the administration of different drugs, such as antivirals and monoclonal antibodies, with most patients showing elevation of AST and ALT levels, and some also of bilirubin, lactate dehydrogenase, and C-reactive protein[55-59]. However, in most cases, elevated levels of AST and ALT do not lead to severe liver injury and the outcome is favorable[59].

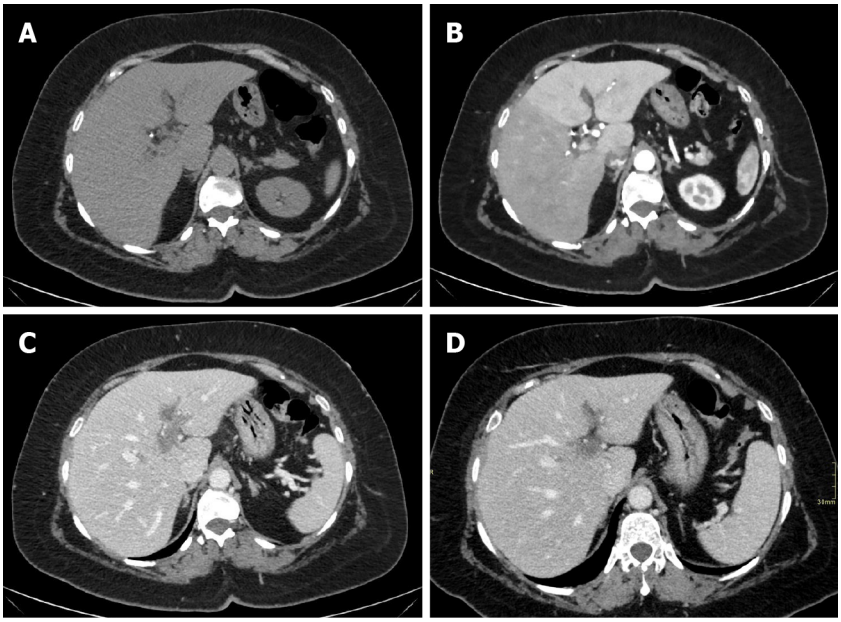
The mechanisms underlying DILI in COVID-19 patients are not yet fully understood and they seem to vary depending on drug type[59,60]. A hepatocellular injury has been more often reported than cholestatic or mixed injury in COVID-19 patients with DILI[59]. On one hand, the drug-induced injury may lead to microvesicular steatosis as a result of drug interference with β-oxidation of fatty acids, mitochondrial respiration, or both which leads to the accumulation of non-esterified fatty acids which are subsequently converted into triglycerides[61,62]. On the other hand, there could be a downregulation of cytochromes p450 or CYPs family enzymes involved in oxidative biotransformation of many drugs, thus altering the metabolism of several COVID-19 drugs[60,61]. DILI could be enhanced by the production of reactive oxygen species by inflammatory cells, in addition to immune mechanisms shown in a small subset of DILI cases[63,64].

The radiological manifestations of DILI in COVID-19 patients are non-specific. One of the most frequent radiological signs of DILI is hepatic steatosis[65]. Hepatic steatosis is seen in the US as a bright liver echo pattern with the markedly increased liver to kidney contrast, and on CT as a reduction of liver attenuation below 40 UH as an absolute value or liver attenuation reduced of more than 10 HU compared to the spleen[66]. MRI is the gold standard for detection and quantification of liver steatosis: new quantitative techniques are now available, and others are still being investigated in US and CT[6]. DILI may occasionally lead to acute hepatitis and, therefore, radiological signs in these cases include hepatomegaly with decreased parenchymal enhancement, periportal edema, gallbladder wall thickening, and ascites[29]. However, the role of traditional and new quantitative techniques for assessing hepatic steatosis and liver injury occurring as a manifestation of DILI in COVID-19 is poorly investigated, and it seems quite difficult to analyze: COVID-19 patients may have more commonly hepatic steatosis and liver injury not related to DILI and there are not specific signs allowing us to differentiate between steatosis and liver injury caused by drugs or by other causes (*e.g.*, steatohepatitis, viral infection)[30] (Table 1).

PVT

The pandemic taught us the great impact of COVID-19 infection on the development of coagulation disorders, especially disseminated intravascular coagulation-like massive intravascular clot formation [67]. In this setting, it has been partially explained that the cytokines' cascade and endothelial damage can lead to the development of intravascular coagulation in the whole body, as reported by Cui *et al*[68]: critically ill patients showed a significantly higher incidence of thrombosis, up to 25%.

One of the most important visceral districts to consider in a setting of an altered vascular and endothelial environment is the portal vein, considering its importance in blood drainage from the



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Figure 4 A 40-year-old men with coronavirus disease 2019 infection and marked respiratory symptoms, underwent contrast-enhanced computed tomography due to elevated liver and biliary enzymes. A and B: On unenhanced phase (A) the liver is within normal limits. After the injection of contrast agent, on arterial phase (B) liver parenchyma shows inhomogeneous enhancement, with hypervascularization of the left liver lobe, as in case of transient hepatic attenuation differences; C: On the portal venous phase, the arterial hypervascularization fades to homogeneous enhancement and diffuse thrombosis of the left branch of the portal vein is demonstrated; D: After 6 mo the patient underwent contrast-enhanced computed tomography that demonstrated persistent portal vein thrombosis without venous collaterals.

gastrointestinal tract.

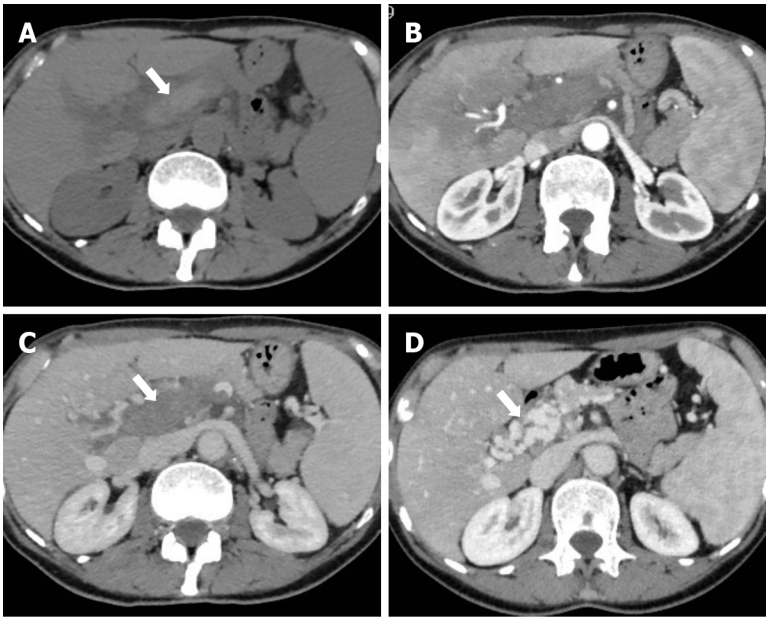
In non-cirrhotic patients, acute PVT may present with pain, even though the majority are found incidentally[69] (Figure 4). Different causes have been demonstrated as key roles in the development of PVT, with infection and inflammation being the most common. Rajani *et al*[70], reported that gastrointestinal inflammation accounts for about 14% of all causes of PVT. As for other viral and bacterial infections, COVID-19 can manifest with different gastrointestinal manifestations, including diarrhea, nausea, vomiting, and abdominal pain, which typically present after the respiratory symptoms (9 d *vs* 7.3 d)[71].

Even if it is well known that the inflammatory environment can lead to the development of micro- and macro-thrombosis, the current literature has not been focused on the impact of PVT in COVID-19 patients. In fact, by searching medical databases, few studies were published regarding this topic, the majority being case reports.

A meta-analysis published in 2020[72] included 18 studies and reported that all COVID-19 patients were over 15-years-old, and the majority were male (62%). The authors found a pooled prevalence of vascular thrombosis of 29.4%, one of the most representative signs in the autoptic series. Similarly, Kheyrandish *et al*[73] (2021) reviewed all cases of PVT published in the literature, confirming the higher incidence in males during infection and in females after vaccination. Thrombocytopenia was the most common laboratory finding, followed by high D-Dimer values, and abnormal coagulation tests.

Radiology plays a key role in the diagnosis of PVT. The first imaging technique useful to determine portal vein patency is US, which can be performed at bedside, especially in critically ill or isolated patients. Acute PVT can manifest as the presence of heterogenous material in the portal vein lumen, which can be partial or complete[74]. Occasionally, the portal vein thrombus can be iso- or hypoechoic on US; in this setting, the use of color doppler can support the final diagnosis, showing the lack of flow in all or some parts of the portal vein lumen[75]. Nowadays, CT is the reference standard imaging technique to evaluate PVT and its extension, both intra-hepatic and into the whole mesenteric venous system. On the unenhanced images, a higher attenuation into the portal vein lumen, due to the fresh clot, can be appreciated. The injection of an intravenous iodinated contrast agent is necessary to evaluate the lack of enhancement in the lumen, more evident in the portal-venous phase. Liver enhancement can be inhomogeneous due to areas of hypervascularization during the arterial phase, then becoming homogenous in the portal-venous and delayed phases[74]. The portal trunk can be dilated, and, sometimes, it is possible to appreciate the enhancement of vein walls due to inflammatory response[76].

Due to the different imaging spectrum of COVID-19 infection, patients may undergo MRI of the upper abdomen. In these settings, acute PVT is represented by an inhomogeneous intraluminal area(s) both on T1- and T2-weighted sequences. On T1-weighted sequences, PVT can manifest as hyperintense to the muscle if it is recent (acute), while isointense if subacute. On T2-weighted sequences PVT can



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Figure 5 A 62-year-old woman with acute portal vein thrombosis after coronavirus disease 2019 infection. A-C: Computed tomography (CT) images show acute thrombosis with hyperintense thrombus on unenhanced phase (A, arrow), heterogeneous enhancement of the liver parenchyma on hepatic arterial phase (B), and complete portal vein thrombosis on portal venous phase (C, arrow); D: Contrast-enhanced CT at 6-mo follow-up demonstrates chronic findings of portal cavernoma with multiple collateral vessels at the hepatic hilum (arrow).

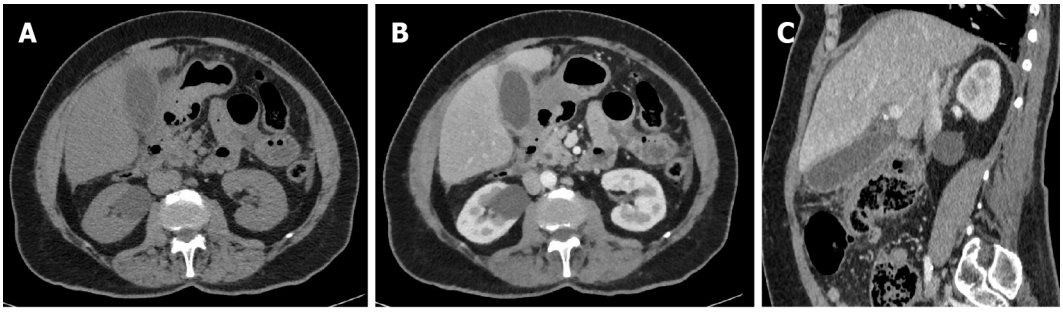
manifest with different grades of hyperintense signal according to the phase (acute or subacute). If MRI is performed also after intravenous injection of contrast agents, the appearance is superimposable to the above-mentioned CT scan (Table 1).

If the PVT is not treated, cavernous transformation can occur (Figure 5): the main portal venous trunk is not appreciable and the development of periportal venous collaterals can help the drainage of venous flow from the gastrointestinal tract to the liver. Considering that chronic PVT is not reported in any patients with COVID19 infection, its findings are out of the scope of the present review.

Biliary and gallbladder involvement

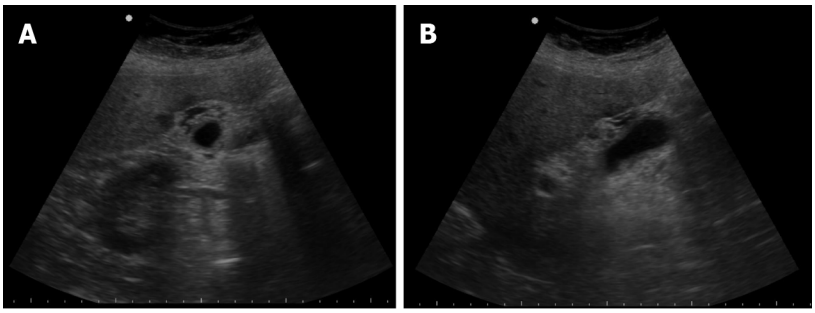
The development of biliary injury in patients with COVID-19 represents an important complication, associated with poor prognosis and clinical outcome[77]. Biliary involvement in patients with COVID-19 often demonstrates clinical and biochemical features similar to sclerosing cholangitis in critically ill patients (SSC-CIP), manifesting as increased cholestasis indexes (GGT and total bilirubin) in patients with prolonged admission to the ICU and no history of biliary or liver disease nor signs of mechanical obstruction[77,78]. Severe cholestasis has been reported in up to 27% of patients with COVID-19 admitted to the ICU[79]. The mechanism of the cholestatic injury in patients with COVID-19 is not completely understood and it is likely multifactorial, with direct viral damage due to the expression of ACE2 on cholangiocytes, immune or inflammatory damage associated with liver injury, toxic bile injury, and ischemic or hypoxic injury of the biliary epithelium[80]. Cholangiopathy has also been observed after chronic exposure to ketamine, a general anesthetic used for sedation of patients with COVID-19 and acute respiratory distress syndrome[81]. The prognosis of patients with SSC-CIP is poor, with mortality in up to 50% of cases due to the development of biliary complications and worsening of liver function[82]. Particularly, patients with pre-existing chronic liver disease have an increased risk of SSC-CIP and higher mortality during COVID-19 infection[83].

Imaging is important to guide the diagnosis of cholangiopathy in patients with COVID-19 in conjunction with laboratory markers and to exclude other causes of biliary obstruction. US and CT can be performed as first-line imaging examinations and can reveal the presence of bile duct dilatation with intrahepatic stones[84] (Figures 6 and 7). Heterogeneous enhancement of the liver parenchyma with periportal edema can also be observed on contrast-enhanced CT[85]. MRI with MRCP should be performed in patients with persistent cholestasis and elevated liver function tests to assess the extension of biliary damage. MRCP can demonstrate features of secondary sclerosing cholangitis characterized by multifocal biliary strictures alternated with dilated tracts, with a "beaded" appearance[79,85]. Biliary strictures can be complicated by biliary cast, presenting as intraductal filling detects on MRCP and T2-weighted images with corresponding linear hyperintensity on unenhanced T1W images. In a recent study by Ghafoor *et al*[86], MRCP findings associated with COVID-19 cholangiopathy included intrahepatic bile duct strictures associated with upstream dilatation in 58% of patients and the presence of biliary casts in 11.8% of cases (Figure 8). Peribiliary changes characterized by hyperintensity on T2W



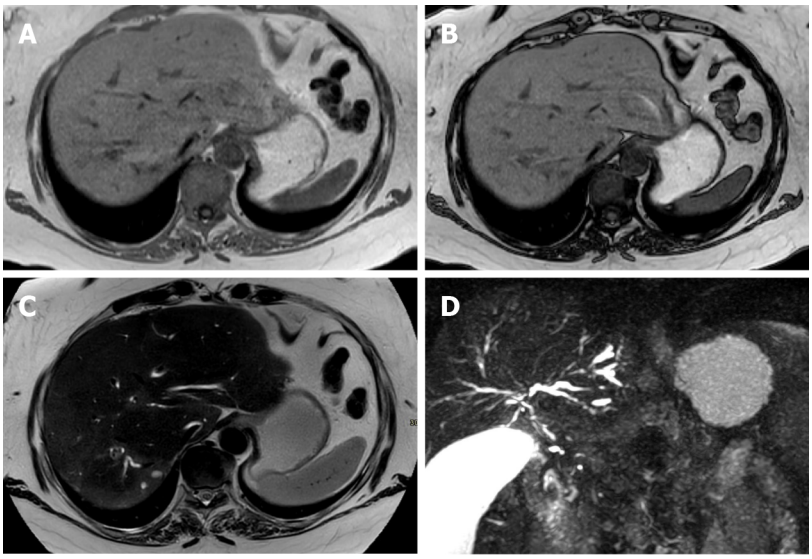
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Figure 6 A 66-year-old men with coronavirus disease 2019 infection, associated with abdominal pain in the upper quadrants. A-C: Contrast-enhanced computed tomography showed diffuse thickening of the gallbladder walls (A), with homogeneous contrast enhancement (B), better appreciable on the sagittal reconstruction (C). The gallbladder lumen is filled with biliary sludge (A) without calcified stones. The final diagnosis was acute acalculous cholecystitis.



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Figure 7 A 53-year-old woman with coronavirus disease 2019 infection and markedly elevated biliary enzymes and C reactive protein levels. A and B: Ultrasonography demonstrates diffuse thickening of the gallbladder wall, associated with multiple small anechoic components into the walls, as in case of intramural abscesses. No stone is appreciated. The final diagnosis was acute acalculous cholecystitis.



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Figure 8 A 44-year-old woman with coronavirus disease 2019 infection underwent magnetic resonance cholangiopancreatography due to elevated biliary enzymes with negative findings on ultrasonography and contrast-enhanced computed tomography. A and B: In-phase (A) and out-of-phase (B) imaging demonstrates hepatic homogeneous parenchyma without areas of fatty infiltration; C: On the T2-weighted image liver inflammation, especially in the right lobe, is characterized by areas of slight hyperenhancement, located nearby the biliary ducts; D: Finally, magnetic resonance cholangiopancreatography allows the evaluation of the biliary tree, showing multiple and diffuse focal biliary stenosis, in particular in the right lobe, with upstream dilation of small biliary ducts. This radiological aspect is typical of sclerosing cholangitis and the final diagnosis was a biliary involvement due to severe acute respiratory syndrome coronavirus 2.

images and DWI restriction were reported in 70.6% of patients, while peribiliary enhancement was observed in 23.1% of cases[86]. Extrahepatic bile duct involvement is rare[86]. Other complications include sepsis with the possible development of hepatic abscesses and progressive liver disease with morphologic features of biliary cirrhosis. Endoscopic retrograde cholangiopancreatography can be performed to confirm the diagnosis of biliary strictures and stones in selected cases and allow treatment of biliary obstruction.

Acalculous cholecystitis has been reported as the most common gallbladder involvement in patients with COVID-19[87,88]. Despite the pathogenesis of acalculous cholecystitis in COVID-19 is still under investigation, the presence of SARS-Cov-2 virus was demonstrated in samples from the gallbladder wall, probably due to the presence of ACE2 receptors in the gallbladder[87]. Other possible causes include mechanical ventilation and prolonged total parenteral nutrition[89]. The US is the first-line imaging modality in patients with suspected acalculous cholecystitis and it may reveal thickened gallbladder wall with peri-cholecystic fluid collection and gallbladder distension, in the absence of gallstones. Contrast-enhanced CT may be performed in case of suspected complications, such as gallbladder perforation, fistula, or necrosis (*i.e.* gangrenous cholecystitis)[90]. CT findings include distended gallbladder with wall thickening, hyperenhancement of the gallbladder wall during postcontrast phases, and pericholecystic fluid[91] (Table 1).

Chronic findings

The current literature is lacking studies evaluating the chronic findings of COVID-19 on liver imaging. The type of hepatic chronic findings should be related to the sequelae of acute liver damage during COVID-19 infection after recovering from the acute disease. Severe liver cholestatic injury can progress into chronic liver disease with the development of cirrhosis, manifesting as abnormal liver morphology with associated imaging features of portal hypertension and ascites. On US, a prospective multiparametric assessment of post-COVID-19 patients observed increased liver stiffness and steatosis at 3-9 mo after COVID-19 compared to normal controls[30]. Complete PVT can progress to portal vein cavernoma if not promptly recanalized and resulting in chronic findings of noncirrhotic portal hypertension and risk of variceal bleeding[49].

Further studies are still needed to assess the evolution of hepatic findings and the possible long-term sequelae of liver damage in patients recovering from COVID-19.

CONCLUSION

Even if COVID-19 is extensively reported as a disease that mainly affects the lungs, the viral infection may cause an involvement of abdominal parenchymal organs and the gastrointestinal tract, increasing the risk of both acute and long-term health problems, especially of liver parenchyma. The liver damage may be caused by different mechanisms, including direct hepatocytes involvement from the viral infection, indirect response to the systemic inflammatory status, or hepatotoxicity due to drugs used to manage the infection. In this setting, the diagnostic imaging workup plays a crucial role for early detection of liver manifestations and assessment of long-term complications.

US should be considered as the main diagnostic option for the first evaluation of liver, biliary tree, and vascular district, in patients with abdominal symptoms, or with altered blood test, while abdominal contrast enhanced CT seems to be the most useful diagnostic tool for the overall abdominal assessment offering useful information regarding not only the liver itself, but also other parenchymal organs and vascular system. Finally, MRI should be considered the tool that better clarifies liver alterations in patients with COVID infection deemed indeterminate on US and CT.

It's important to underline that the main limitation in this field which should still be considered is the difficulty to understand the main COVID-19 pathological mechanisms and their related consequences. Further studies should be more focused on the evaluation of COVID-19 patients, in particular those with liver involvement, to quickly address the diagnosis and the best management possible.

A comprehensive knowledge of COVID-19 hepatic involvement assessed through the different diagnostic imaging modalities can help clinicians in addressing the correct treatment and long-term management of the disease.

FOOTNOTES

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Country/Territory of origin: Italy

ORCID number: Davide Ippolito 0000-0002-2696-7047; Cesare Maino 0000-0002-5742-802X; Federica Vernuccio 0000-0003-0350-1794; Roberto Cannella 0000-0002-3808-0785; Riccardo Inchingolo 0000-0002-0253-5936; Michele Dezio 0000-0001-6491-4969; Riccardo Faletti 0000-0002-8865-8637; Pietro Andrea Bonaffini 0000-0001-5335-9429; Marco Gatti 0000-0001-8168-5280; Sandro Sironi 0000-0002-4469-5073.

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ORIGINAL ARTICLE

Basic Study

Saccharomyces cerevisiae prevents postoperative recurrence of Crohn's disease modeled by ileocecal resection in HLA-B27 transgenic rats

Caroline Valibouze, Silvia Speca, Caroline Dubuquoy, Florian Mourey, Lena M'Ba, Lucil Schneider, Marie Titecat, Benoît Foligné, Michaël Genin, Christel Neut, Philippe Zerbib, Pierre Desreumaux

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Caroline Valibouze, Lena M'Ba, Lucil Schneider, Philippe Zerbib, Department of Digestive Surgery and Transplantation, Lille University Hospital, Lille 59037, France

Caroline Valibouze, Silvia Speca, Marie Titecat, Benoît Foligné, Christel Neut, Philippe Zerbib, Pierre Desreumaux, U1286 - INFINITE - Institute for Translational Research in Inflammation, Univ. Lille, Inserm, CHU Lille, Lille 59000, France

Caroline Dubuquoy, Intestinal Biotech Development, Lille 59045, France

Florian Mourey, Department of Research and Applications, Gnosis by Lesaffre, Lesaffre Group, Marcq-en-Baroeul 59700, France

Michaël Genin, ULR 2694 - METRICS: Évaluation des Technologies de Santé et des Pratiques Médicales, University of Lille, Lille University Hospital, Lille 59000, France

Pierre Desreumaux, Department of Hepato-Gastroenterology, Lille University Hospital, Lille 59037, France

Corresponding author: Caroline Valibouze, MD, Surgeon, Department of Digestive Surgery and Transplantation, Lille University Hospital, Rue Michel Polonovski, Lille 59037, France.
caroline.valibouze@chu-lille.fr

Abstract

BACKGROUND

Postoperative recurrence (POR) after ileocecal resection (ICR) affects most Crohn's disease patients within 3-5 years after surgery. Adherent-invasive *Escherichia coli* (AIEC) typified by the LF82 strain are pathobionts that are frequently detected in POR of Crohn's disease and have a potential role in the early stages of the disease pathogenesis. *Saccharomyces cerevisiae* CNCM I-3856 is a probiotic yeast reported to inhibit AIEC adhesion to intestinal epithelial cells and to favor their elimination from the gut.

AIM

To evaluate the efficacy of CNCM I-3856 in preventing POR induced by LF82 in an HLA-B27 transgenic (TgB27) rat model.

METHODS

Sixty-four rats [strain F344, 38 TgB27, 26 control non-Tg (nTg)] underwent an ICR at the 12th wk (W12) of life and were sacrificed at the 18th wk (W18) of life. TgB27 rats were challenged daily with oral administration of LF82 (10^9 colony forming units (CFUs)/day (d), n = 8), PBS (n = 5), CNCM I-3856 (10^9 CFUs/d, n = 7) or a combination of LF82 and CNCM I-3856 (n = 18). nTg rats receiving LF82 (n = 5), PBS (n = 5), CNCM I-3856 (n = 7) or CNCM I-3856 and LF82 (n = 9) under the same conditions were used as controls. POR was analyzed using macroscopic (from 0 to 4) and histologic (from 0 to 6) scores. Luminal LF82 quantifications were performed weekly for each animal. Adherent LF82 and inflammatory/regulatory cytokines were quantified in biopsies at W12 and W18. Data are expressed as the median with the interquartile range.

RESULTS

nTg animals did not develop POR. A total of 7/8 (87%) of the TgB27 rats receiving LF82 alone had POR (macroscopic score ≥ 2), which was significantly prevented by CNCM I-3856 administration [6/18 (33%) TgB27 rats, P = 0.01]. Macroscopic lesions were located 2 cm above the anastomosis in the TgB27 rats receiving LF82 alone and consisted of ulcerations with a score of 3.5 (2 - 4). Seven out of 18 TgB27 rats (39%) receiving CNCM I-3856 and LF82 had no macroscopic lesions. Compared to untreated TgB27 animals receiving LF82 alone, coadministration of CNCM I-3856 and LF82 significantly reduced the macroscopic [3.5 (2 - 4) *vs* 1 (0 - 3), P = 0.002] and histological lesions by more than 50% [4.5 (3.3 - 5.8) *vs* 2 (1.3 - 3), P = 0.003]. The levels of adherent LF82 were correlated with anastomotic macroscopic scores in TgB27 rats (r = 0.49, P = 0.006), with a higher risk of POR in animals having high levels of luminal LF82 (71.4% *vs* 25%, P = 0.02). Administration of CNCM I-3856 significantly reduced the levels of luminal and adherent LF82, increased the production of interleukin (IL)-10 and decreased the production of IL-23 and IL-17 in TgB27 rats.

CONCLUSION

In a reliable model of POR induced by LF82 in TgB27 rats, CNCM I-3856 prevents macroscopic POR by decreasing LF82 infection and gut inflammation.

Key Words: Crohn's disease; Recurrence; *Escherichia coli*; Probiotic; *Saccharomyces cerevisiae*; Colorectal surgery

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Core Tip: Gut dysbiosis plays a main role in the postoperative recurrence (POR) of Crohn's disease (CD). CD dysbiosis is characterized by a lower microbiota diversity with an increase in pathogenic species. Among them, adherent-invasive *Escherichia coli* (AIEC) has been linked to POR. *Saccharomyces cerevisiae* (*S. cerevisiae*) CNCM I-3856 is a probiotic yeast that specifically targets AIEC by preventing the bacterial adhesion process and inhibiting its persistence within the bowel. This study confirmed the capacity of *S. cerevisiae* CNCM I-3856 to prevent AIEC-induced POR by decreasing the infection in a transgenic HLA-B27 rat model of POR after ileocecal resection.

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INTRODUCTION

Crohn's disease (CD) is a complex chronic inflammatory bowel disease that requires surgical resection of macroscopic lesions in approximately 30%-50% of patients in their lifetime[1]. Unfortunately, surgery is not curative, and endoscopic recurrence at the anastomotic site occurs in up to 70% of patients in the first year after surgery, followed by clinical recurrence a few years later[2]. Postoperative management of these patients is crucial to identify those at highest risk of recurrence to begin rapid prophylactic treatments targeting mainly tumor necrosis factor α (TNFα)[3], interleukins 12/23 and α4β7 integrins on leukocytes[4]. Given the high rate of recurrence after intestinal resection for CD and the cost and potential adverse effects of biologic therapies used in prophylaxis, there is a clear need to identify the mechanisms leading to postoperative recurrence (POR), to develop noninvasive methods predicting

recurrence and to propose new evidence-based therapeutic strategies.

The physiopathology sustaining POR of CD remains partially unknown. Abnormal interactions between the mucosal/mesenteric immune system and the intestinal microbiota favored by surgical techniques and environmental factors are pivotal hallmarks in POR dynamics[5]. Recently, ileal transcriptome analyses of CD patients found a gene signature of POR characterized by an upregulation of the interleukin (IL)-23 and IL-17 pathways together with abnormal JAK/STAT activation[6]. Numerous changes in the microbial composition and a reduction in species diversity have been observed in the intestinal flora of CD patients[7], and a few studies have identified an intestinal microbial signature associated with POR. Recolonization of the neoterminal ileum by *Escherichia coli* (*E. coli*), Bacteroides, and Fusobacteriaceae and the depletion of Streptococcaceae, Actinomycineae and *Faecalibacterium* are associated with endoscopic recurrence of CD[8]. Among these microorganisms, adherent-invasive *E. coli* (AIEC) isolated more than 20 years ago by Darfeuille-Michaud *et al*[9] from the ileal mucosa of a patient with CD[10] remains one of the most prominent and influential strains associated with CD. AIEC are pathobionts found in approximately 30% of CD patients and in 10% of healthy controls[11]. They are not strictly pathogenic bacteria, and their influence on CD physiopathology remains incompletely understood. However, AIEC is associated with the early stages of CD and is predictive of endoscopic POR at 6 mo[12], reinforcing the need for interventional studies targeting these bacteria to better understand their direct impact on mucosal inflammation and to find new opportunities to treat CD patients.

Several therapeutic strategies, including the use of antibiotics[13], pre/probiotics[14] and fecal microbiota transplantation[15], have been proposed to target the intestinal flora in CD. Due to side effects or limited efficacy, their routine utilization cannot be recommended[16,17]. Other strategies to inhibit adhesion or to specifically erase AIEC using FimH blockers[18,19] or specific bacteriophages[20,21] are ongoing and seem more promising in preclinical studies. In this context, *Saccharomyces cerevisiae* (*S. cerevisiae*) CNCM I-3856 is a probiotic yeast with good tolerance and beneficial effects on gastrointestinal symptoms[22,23] that has been shown to agglutinate the LF82 AIEC strain and to prevent its adhesion to intestinal epithelial cells *in vitro*, favoring LF82 elimination from the gut of mice [24]. Among the thousands of strains belonging to the AIEC family and identified from European and USA isolates, LF82 remains the most studied reference strain that can both adhere to and invade epithelial cells and, moreover, survive and replicate within macrophages without inducing cellular death[25,26].

In the present study, we developed a new animal model of POR of CD occurring 6 wk after ileocecal resection (ICR) in HLA-B27 transgenic (Tg) rats[27,28] infected by the LF82 AIEC strain[29] to better evaluate the causal role of LF82 on the early steps of CD lesions and the effectiveness of a rationally selected *S. cerevisiae* CNCM I-3856 probiotic to prevent recurrence of the disease.

MATERIALS AND METHODS

Animals

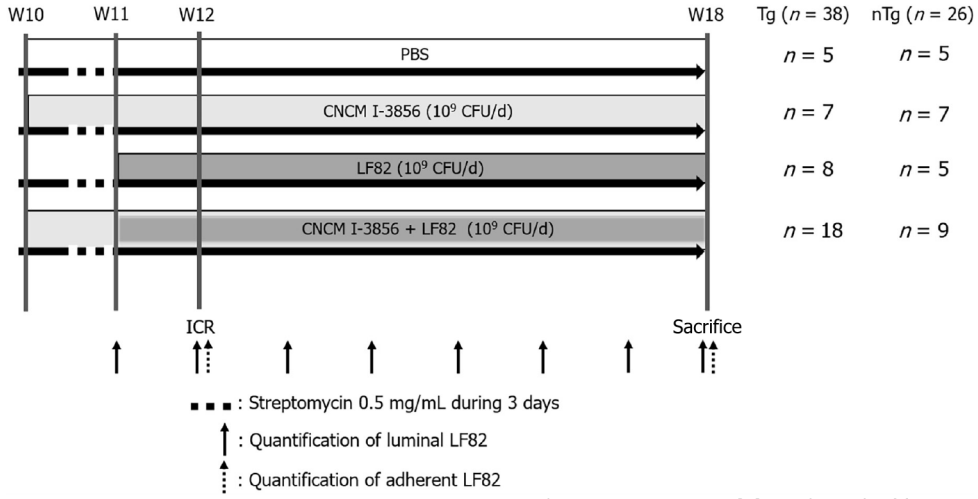
HLA-B27 transgenic (Tg) and nontransgenic (nTg) control Fisher rats (strain F344) were provided by Professor M. Breban (Cochin Institute, INSERM U1016, Paris, France). Sixty-four rats were maintained in a specific pathogen-free facility at the Institut Pasteur (Lille, France) and were fed a standard diet with free access to water. Animals were maintained at a constant temperature with a 12-hour light/dark cycle. Intragastric gavage administration was carried out with conscious animals using straight gavage needles appropriate for the animal size. Surgery was performed under general anesthesia, and postoperative analgesia by opioid treatment was provided. All animals were euthanized by cervical dislocation under general anesthesia. Experiments were realized according to the European directive 2016/63/UE enforced by the decree n°2013-118 and authorized by the departmental ethics committee (No. CEEA 01292-01).

AIEC LF82 and S. cerevisiae CNCM I-3856 strains

The streptomycin-kanamycin-resistant AIEC strain LF82 isolated from an ileal biopsy of a patient with CD was provided by Professor Nicolas Barnich (Clermont-Auvergne University, France) and used as an AIEC reference strain[30]. Bacteria were routinely grown at 37 °C in Brain-Heart broth or on Drigalski agar plates. The dry *S. cerevisiae* CNCM I-3856 yeast strain was provided by Lesaffre International (Marcq-en-Baroeul, France). The LF82 and *S. cerevisiae* CNCM I-3856 strains were rehydrated at room temperature in PBS (pH = 7.2, 2 × 10^9 colony forming units (CFUs)/mL) before gavage.

Experimental design

ICR with end-to-end anastomosis was performed at 12 wk (W) of life (W12) in 64 rats (38 Tg, 26 nTg) (Figure 1). ICR was performed blindly by two operators (Caroline Dubuquoy and Caroline Valibouze) in Tg and nTg animals. Tg rats were challenged daily by oral gavage in the morning with PBS (n = 5), *S. cerevisiae* CNCM I-3856 alone (10^9 CFUs/day (d)) (n = 7), LF82 alone (10^9 CFUs/d) in the afternoon (n = 8), or the combination of *S. cerevisiae* CNCM I-3856 (10^9 CFUs/d) and LF82 (10^9 CFUs/d) (n = 18) given



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Figure 1 Study design. HLA-B27 transgenic rats (Tg) and wild-type rats (nTg) were randomized to receive phosphate buffered saline (*n* = 10), *Saccharomyces cerevisiae* (*S. cerevisiae*) CNCM I-3856 (*n* = 14), adherent-invasive *Escherichia coli* strain LF82 (*n* = 13), or *S. cerevisiae* CNCM I-3856 and LF82 (*n* = 27) by oral gavage from week (W) 10 or 11 to W18. Ileocecal resection was performed at W12, and animals were sacrificed at W18. Streptomycin (dotted line) was given on the last 3 d of W10 in all rats. Luminal (arrows) and/or adherent (dotted arrows) LF82 was quantified weekly during the 8-wk study. CFU: Colony-forming unit; PBS: Phosphate buffered saline; ICR: Ileocecal resection; d: Day.

in the morning and in the afternoon, respectively. Age-matched nTg rats receiving PBS (*n* = 5), *S. cerevisiae* CNCMI-3856 alone (*n* = 7), LF82 alone (*n* = 5), or the combination of *S. cerevisiae* CNCM I-3856 and LF82 (*n* = 9) under the same conditions were used as controls. LF82 was administered from W11 to W18, and *S. cerevisiae* CNCM I-3856 was similarly administered from W10 to W18 in Tg and nTg rats. Streptomycin was given in drinking water at 0.5 mg/mL for the last 3 d of W10 in Tg and control animals. The rats were followed during the eight-week procedure for weight changes (% of change compared to initial body weight at W11), diarrhea and the presence of macroscopic bloody stools and were killed at W18.

Macroscopic and histologic lesions

At W18, the whole intestine was excised and photographed. Anastomotic macroscopic lesions (± 2 cm above anastomosis) were assessed blindly using a macroscopic grading scale adapted from the Rutgeerts score ranging from 0 to 4 (Figure 2)[2]. By analogy with endoscopic recurrence after surgery in patients with CD (25), POR was defined by a macroscopic score of ≥ 2 corresponding to the presence of ulcerations ± stenosis. The results were expressed as the median with the interquartile range (IQR).

Transparietal biopsies of anastomotic areas were collected during surgery at W12 and W18. Tissues were fixed in 4% buffered formaldehyde, embedded in paraffin and stained by May-Grunwald Giemsa for scoring (from 0 to 6) using the adapted score of Geboes (Table 1)[31]. Identical areas of each section of the different biopsy specimens were examined at 10× magnification by two blinded observers familiar with the scoring system (Caroline Dubuquoy and Caroline Valibouze). Anastomotic histologic scores were expressed as the median score with IQR when an interobserver coefficient of variation < 15% was obtained.

Luminal and adherent quantification of LF82

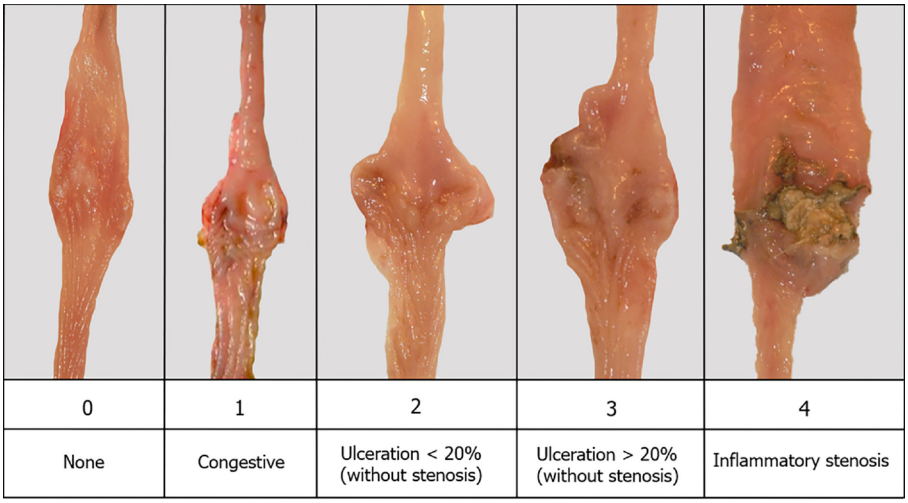
Feces (10 - 600 mg) were collected weekly from W11 to W18 for each animal after abdominal massage for the quantification of luminal LF82. Mucosal anastomotic swabs (10 - 100 mg) were performed at W12 during surgery and at sacrifice (W18) in all animals for the quantification of anastomotic adherent LF82. Fresh feces and swabs were collected in 1.5 mL of sterile cysteinated Ringer's solution. After serial dilutions, samples were incubated for 24 - 48 h at 37 °C in Drigalski agar containing 100 μg/mL streptomycin to select and quantify LF82 expressed as log10 CFUs *per* gram of feces. The results are expressed as the median with the IQR.

mRNA quantification in anastomotic biopsies at W12 and W18

Anastomotic biopsies were frozen at -80 °C, and total RNA was extracted using a Nucleospin RNA kit (Macherey Nagel). After RNAse inactivation, genomic DNA was suppressed from the samples by DNAse treatment, and total RNA was extracted in RNAse-free water. The RNA content was measured using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Retro-transcription of total RNA was achieved using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Random primers, RT buffer and reverse transcriptase were added to 1 μg of

Table 1 Anastomotic histologic score (0-6)

Score	Histologic lesions
0	None
1	Inflammatory infiltrate and mucosal erosions < 30% of the section
2	30% < inflammatory infiltrate and mucosal erosions < 70% of the section
3	Inflammatory infiltrate and mucosal erosions > 70% of the section
4	Mucosal ulceration < 30% of the section
5	30% < mucosal ulceration < 70% of the section
6	Mucosal ulceration > 70% of the section



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Figure 2 Anastomotic macroscopic score (0-4).

total RNA, and the samples were incubated for 10 min at 25 °C, then 2 h at 37 °C and finally 5 min at 85 °C in the Gene AmpPCR System 9700 automaton (Thermos Fisher Scientific, Waltham, Massachusetts, USA). All kits were used according to the manufacturers' protocols. IL-1β, IL-6, TNFα, interferon (IFN)γ, IL-17, IL-23 and IL-10 were quantified by quantitative polymerase chain reaction (PCR) in real time for 40 cycles in the StepOnePlus™ Real Time PCR system (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using SYBR Green PCR Master Mix (Thermo Fisher Scientific). qPCR signal quantification was expressed relative to the expression of β-actin as the reference gene. The results are expressed as the median with IQR.

Statistical analysis

Data are expressed as the median with IQR. Comparisons were performed using the nonparametric Mann-Whitney test for unmatched data and the Wilcoxon signed-rank test for matched data. Pearson's chi-square test was used for contingency analysis. The correlation between macroscopic scores and the number of LF82 was tested using Spearman's test. To classify animals with low or high quantities of LF82, a cutoff value was determined using the receiver operating characteristic (ROC) curve. The risk of recurrence for low and high producers was compared using Pearson's chi-square test. All statistical tests were two-tailed and considered statistically significant if $P < 0.05$. Statistical analyses were conducted using the GraphPad Prism 5.00 (GraphPad Software, San Diego, CA) software package for PCR and Xlstat 2020.1 version for the ROC curve.

RESULTS

Effect of S. cerevisiae CNCM I-3856 on clinical signs

No mortality, diarrhea or bloody stools were observed in any animals receiving PBS, LF82 alone, *S. cerevisiae* CNCM I-3856 alone or *S. cerevisiae* CNCM I-3856 and LF82 during the 8-wk observation study.

A similar pattern of weight evolution was observed in nTg (Figure 3A) and Tg animals (Figure 3B), with significant weight loss occurring one week after surgery followed by a weight recovery phase. More important weight loss was transiently observed at W13 in Tg rats receiving LF82 *vs S. cerevisiae* CNCM I-3856 and LF82 (95.7, IQR: 92 - 97 *vs* 85.4, IQR: 81 - 94, $P = 0.007$). The global weight changes assessed by the relative difference in weight variation between W11 and W18 were similar in the 4 groups of Tg and nTg animals.

Effect of S. cerevisiae CNCM I-3856 on macroscopic anastomotic lesions and POR

No intestinal lesions were present at W12 in any animal. No macroscopic lesions (or therefore POR) were observed at W18 in control nTg animals receiving PBS, *S. cerevisiae* CNCM I-3856 alone, LF82 alone, or *S. cerevisiae* CNCM I-3856 and LF82 (Figure 4A). In contrast, anastomotic macroscopic lesions corresponding mainly to edema and ulcerations on more than 20% of the anastomotic area without stenosis were observed in Tg rats receiving LF82 (3.5, IQR: 2 - 4), leading to 87.5% POR in this group of animals (Figure 4A and B). Compared to untreated Tg rats receiving LF82 (3.5, IQR: 2 - 4), coadministration of *S. cerevisiae* CNCM I-3856 and LF82 significantly reduced the macroscopic score (1, IQR: 0 - 2, $P = 0.002$) and POR (87.5% *vs* 33.3%, $P = 0.01$) by more than 60% (Figure 4A and B). Anastomotic macroscopic lesions were similar in Tg rats receiving PBS or *S. cerevisiae* CNCM I-3856 alone or *S. cerevisiae* CNCM I-3856 and LF82, without a difference compared to those of control nTg animals (Figure 4A).

Effect of S. cerevisiae CNCM I-3856 on anastomotic histologic lesions

No histologic lesions were present at W12 in any animal (data not shown). At W18, no significant and only mild histologic lesions characterized by neutrophil infiltration not exceeding 30% of lamina propria cells were observed in control nTg animals receiving either PBS, LF82 alone, *S. cerevisiae* CNCM I-3856 alone or *S. cerevisiae* CNCM I-3856 and LF82 (Figure 5). In contrast, erosions and mucosal ulcerations associated with moderate neutrophil infiltration were observed at W18 in Tg rats receiving LF82 (4.5, IQR: 3.3 - 5.8) (Figure 5). Compared to untreated Tg animals receiving LF82, coadministration of *S. cerevisiae* CNCM I-3856 and LF82 significantly reduced the histological lesions by more than 50% (4.5, IQR: 3.3 - 5.8 *vs* 2, IQR: 1.3-3, $P = 0.003$) (Figure 5). No significant lesions were observed in Tg rats receiving PBS or *S. cerevisiae* CNCM I-3856 alone, which was not different from the findings in control nTg animals (Figure 5).

Effect of CNCM I-3856 on luminal and adherent LF82 Levels (W12-W18)

At W12, *i.e.*, one week after the beginning of LF82 administration (10^9 CFUs/d), the quantities of luminal (Figure 6A) and adherent (Figure 7A) LF82 were similar in Tg and nTg rats receiving LF82 alone or *S. cerevisiae* CNCM I-3856 and LF82. The levels of luminal (4.4, IQR: 2.5 - 5.2 *vs* 3.4, IQR: 1.7 - 5.5) and adherent (2.7, IQR: 2.4 - 3 *vs* 3.1, IQR: 2.3 - 5) LF82 remained similar between W12 and W18 in Tg rats receiving LF82 alone (Figures 6 and 7), while a significant decrease in luminal (4.6, IQR: 3.5 - 5.2 *vs* 1.8, IQR: 1.7 - 2.3, $P = 0.0002$) and adherent (3.1, IQR: 2.5 - 3.6 *vs* 2.5, IQR: 2.3 - 2.6, $P = 0.0005$) LF82 was observed between W12 and W18 in paired Tg animals receiving *S. cerevisiae* CNCM I-3856 and LF82 (Figures 6 and 7).

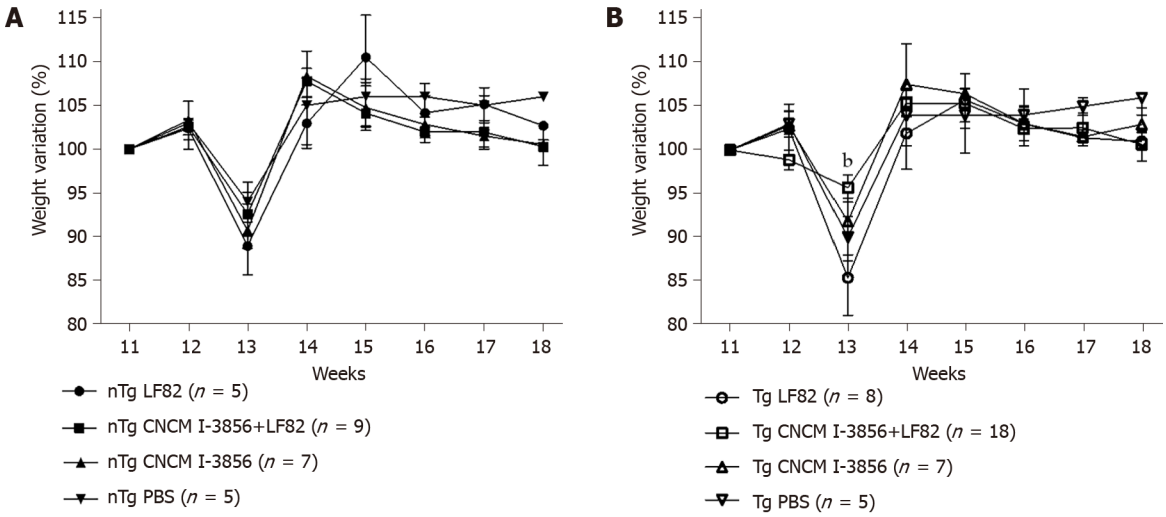
In addition, the global persistence of viable luminal LF82 after surgery and during the last 5 wk of the study was significantly higher in the stools of Tg rats receiving LF82 alone (0.22, IQR: 2.071e-008 - 0.7) compared to Tg rats receiving *S. cerevisiae* CNCM I-3856 and LF82 (-0.6, IQR: -0.7 - 0.3, $P = 0.0004$) (Figure 8).

Correlation between LF82 Levels and macroscopic lesions in Tg rats

A correlation was found between the levels of adherent LF82 and the scores of anastomotic macroscopic lesions observed at W18 in Tg animals receiving LF82 alone or in combination with *S. cerevisiae* CNCM I-3856 ($r = 0.49$, $P = 0.006$) (Figure 9A). These levels of anastomotic adherent LF82 were correlated at W12 ($r = 0.81$, $P = 0.02$) and W18 ($r = 0.79$, $P = 0.03$) with the levels of luminal LF82 in paired Tg animals receiving LF82 alone (Figure 9B and C). Next, we analyzed whether luminal LF82 Levels at W14 may be predictive of POR in the 26 Tg rats receiving LF82 alone ($n = 8$) or in combination with *S. cerevisiae* CNCM I-3856 ($n = 18$). Using a cutoff value of 2.262 Log10 CFUs of luminal LF82 per gram of stool determined by the ROC curve, 14 animals at W14 were classified as highly infected by LF82, and 12 were classified as mildly infected (Figure 10A). POR was significantly more frequent in the highly infected Tg rats than in the mildly infected Tg rats (71.4% *vs* 25%, $P = 0.02$) (Figure 10B). A value of 2.262 Log10 CFUs luminal LF82 *per* gram of stool at W14 had an 80% sensitivity, 69.2% specificity, 71.4% positive predictive value and 75% negative predictive value for POR.

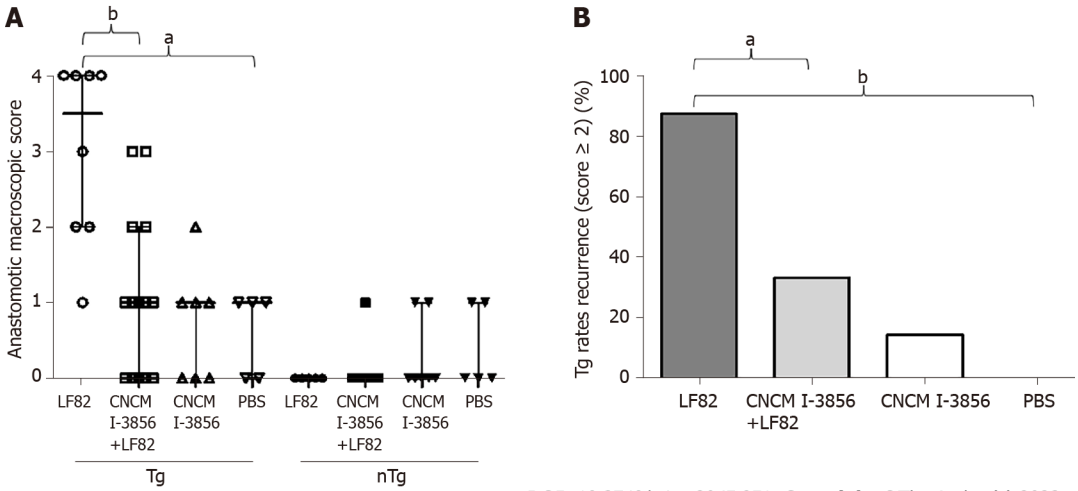
Anastomotic cytokine mRNA quantification

The levels of IL-1β, IL-6, TNFα and IFNγ mRNA were variable and similar in all Tg and nTg animals at W12 and W18, regardless of the presence of POR, LF82 administration or treatment with *S. cerevisiae* CNCM I-3856 (data not shown).



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Figure 3 Body weight evolution. A: Evolution of weight changes compared to body weight at W11 in nontransgenic (nTg) rats; B: Evolution of weight changes compared to body weight at W11 in transgenic (Tg) rats. $^{b}P < 0.01$. LF82: Adherent-invasive *Escherichia coli* strain LF82; CNCM I-3856: *Saccharomyces cerevisiae* CNCM I-3856; PBS: Phosphate buffered saline.



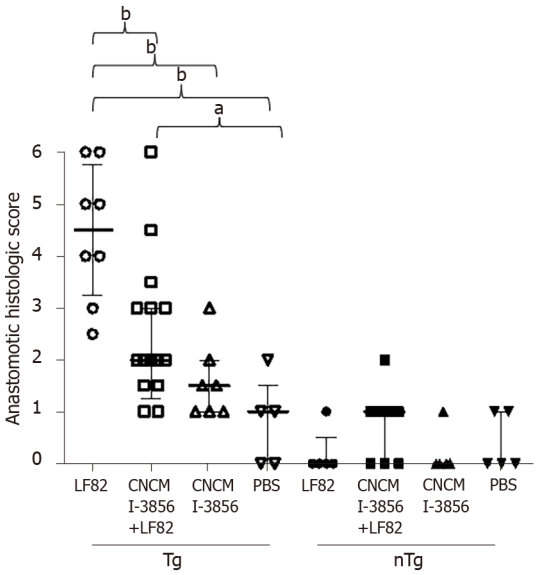
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Figure 4 Anastomotic macroscopic lesions and postoperative recurrence at sacrifice. A: Anastomotic macroscopic scores in the different groups of HLA-B27 transgenic (Tg) rats and wild-type (nTg) rats at sacrifice; B: % postoperative recurrence (anastomotic macroscopic score ≥ 2) at sacrifice in HLA-B27 Tg rats. $^{a}P < 0.05$, $^{b}P < 0.01$. LF82: Adherent-invasive *Escherichia coli* strain LF82; CNCM I-3856: *Saccharomyces cerevisiae* CNCM I-3856; PBS: Phosphate buffered saline.

Concerning IL-10 mRNA levels, the only significant difference found by paired analysis in the different groups of animals revealed higher levels of IL-10 mRNA at W18 compared to W12 in animals receiving *S. cerevisiae* CNCM I-3856. In the Tg groups, administration of *S. cerevisiae* CNCM I-3856 with or without the coadministration of LF82 induced a significant increase in IL-10 production between surgery and sacrifice (2.5×10^5, IQR: 1.7×10^5 - 2.6×10^5 *vs* 4.9×10^5, IQR: 3.3×10^5 - 9×10^5, $P = 0.017$ and 2.6×10^5, IQR: 1.5×10^5 - 3.9×10^5 *vs* 7.4×10^5, IQR: 5.3×10^5 - 0.4×10^5, $P = 0.031$, respectively), while similar IL-10 Levels were found in animals receiving LF82 alone (Figure 11A-C).

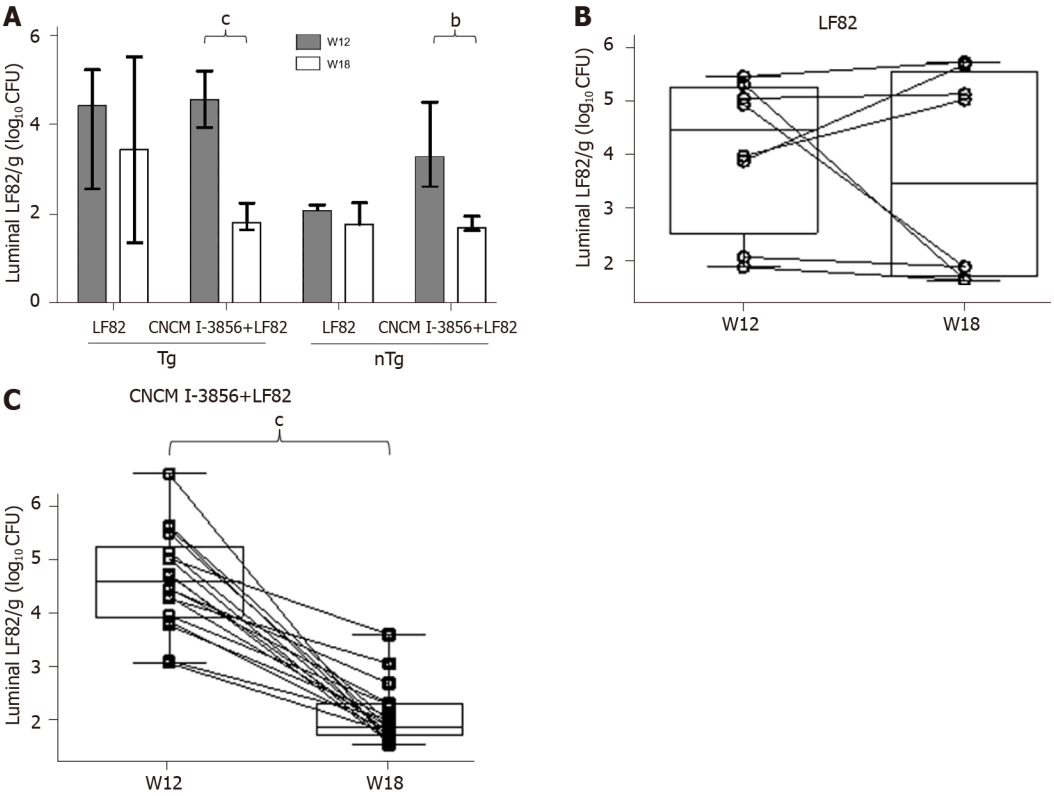
Concerning IL-23 mRNA levels, a significant increase was observed at W18 in Tg animals receiving LF82 alone in comparison to the groups of rats treated with *S. cerevisiae* CNCM I-3856 with or without administration of LF82 ($P = 0.04$ and $P = 0.006$, respectively) (Figure 12A). Additionally, using a paired t test, a significant increase in inflammatory IL-23 production was observed between surgery and sacrifice in the Tg group receiving LF82 alone (2.2×10^4, IQR: 1.8×10^4 - 8×10^4 *vs* 26.9×10^4, IQR: 6.1×10^4 - 6×10^4, $P = 0.008$), while no significant difference was observed in the Tg groups treated with *S. cerevisiae* CNCM I-3856 with or without administration of LF82 (Figure 12B-D).

Analysis of IL-17 mRNA levels found significantly higher rates at W18 in Tg rats receiving LF82 in comparison with the Tg group receiving *S. cerevisiae* CNCM I-3856 and LF82 (2.7×10^4, IQR: 0.8×10^4 - $9,5 \times 10^4$ *vs* 0.4×10^4, IQR: 0.2×10^4 - 0.6×10^4, $P = 0.015$) (Figure 13).



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Figure 5 Anastomotic histologic lesions at sacrifice. Anastomotic histologic scores in the different groups of HLA-B27 transgenic (Tg) rats and wild-type (nTg) rats at sacrifice. [a]*P* < 0.05, [b]*P* < 0.01. LF82: Adherent-invasive *Escherichia coli* strain LF82; CNCM I-3856: *Saccharomyces cerevisiae* CNCM I-3856; PBS: Phosphate buffered saline.

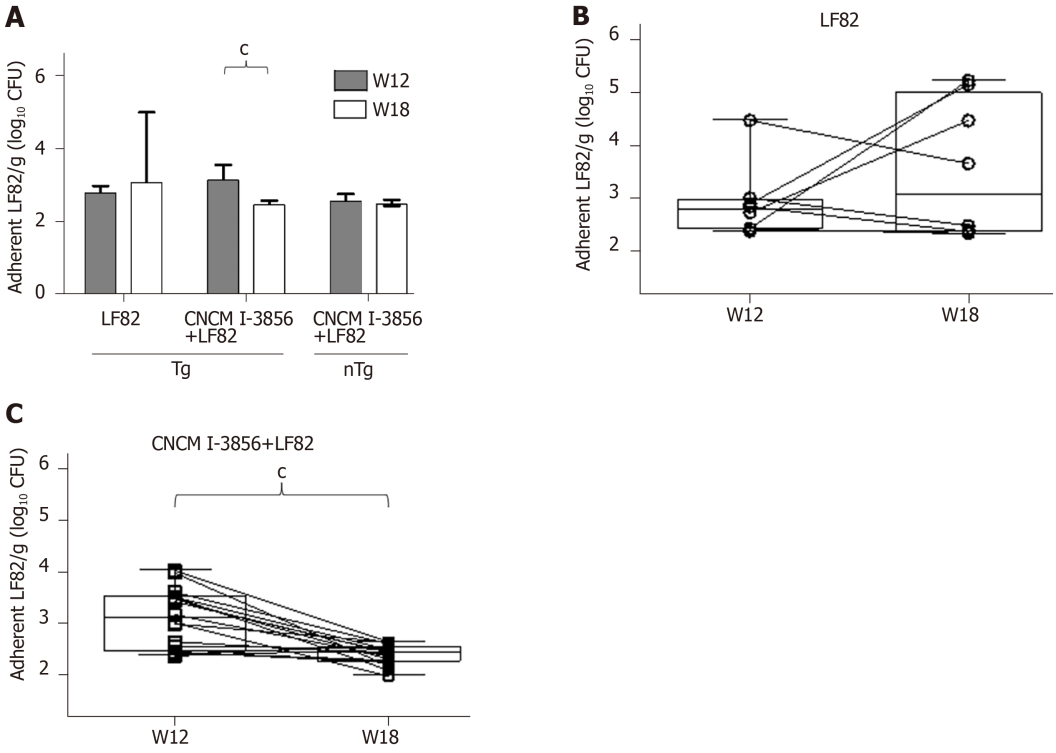


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Figure 6 Levels of luminal adherent-invasive *Escherichia coli* LF82 at surgery and sacrifice. A: Luminal levels of adherent-invasive *Escherichia coli* strain LF82 at surgery [week (W) 12] and sacrifice (W18) in the different groups of HLA-B27 transgenic (Tg) rats and wild-type (nTg) rats; B: Luminal levels of LF82 at W12 and W18 in paired Tg rats receiving LF82 alone; C: Luminal levels of LF82 at W12 and W18 in paired Tg rats receiving *Saccharomyces cerevisiae* CNCM I-3856 and LF82. [b]*P* < 0.01, [c]*P* < 0.001. CFU: Colony-forming unit; log10: Decimal logarithm.

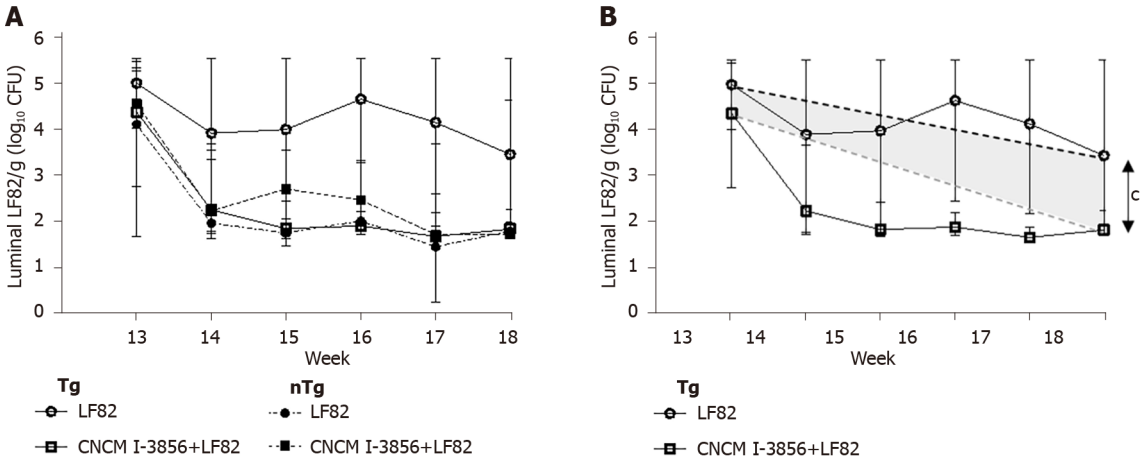
DISCUSSION

The role of the intestinal microbiota composition and diversity in POR of CD is important. Among intestinal microorganisms potentially involved in POR, many studies support the roles of AIEC in early



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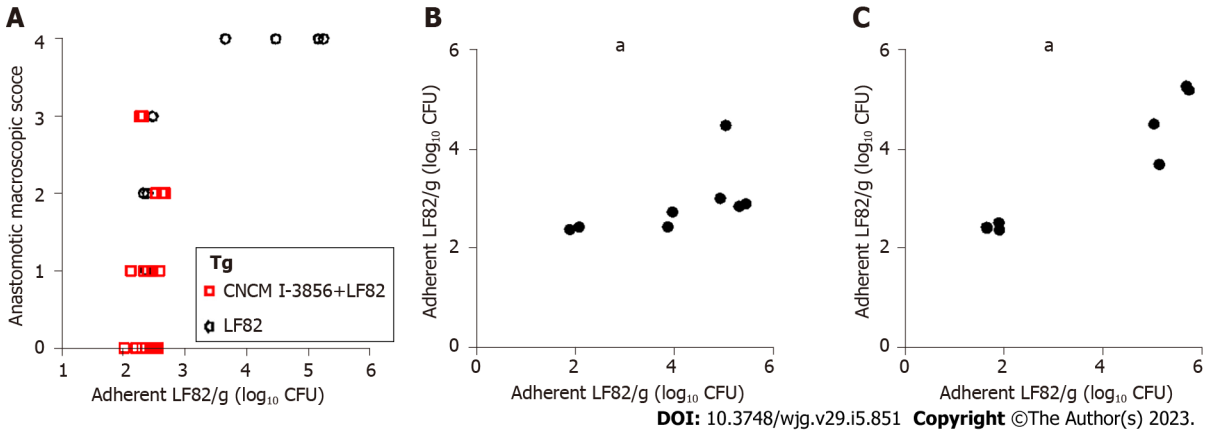
Figure 7 Levels of anastomotic adherent adherent-invasive *Escherichia coli* LF82 at surgery and sacrifice. A: Adherent levels of adherent-invasive *Escherichia coli* strain LF82 at surgery [week (W) 12] and sacrifice (W18) in the different groups of HLA-B27 transgenic (Tg) rats and wild-type (nTg) rats; B: Adherent levels of LF82 at W12 and W18 in paired Tg rats receiving LF82 alone; C: Adherent levels of LF82 at W12 and W18 in paired Tg rats receiving *Saccharomyces cerevisiae* CNCM I-3856 and LF82. °P < 0.001. CFU: Colony-forming unit; log10: Decimal logarithm.



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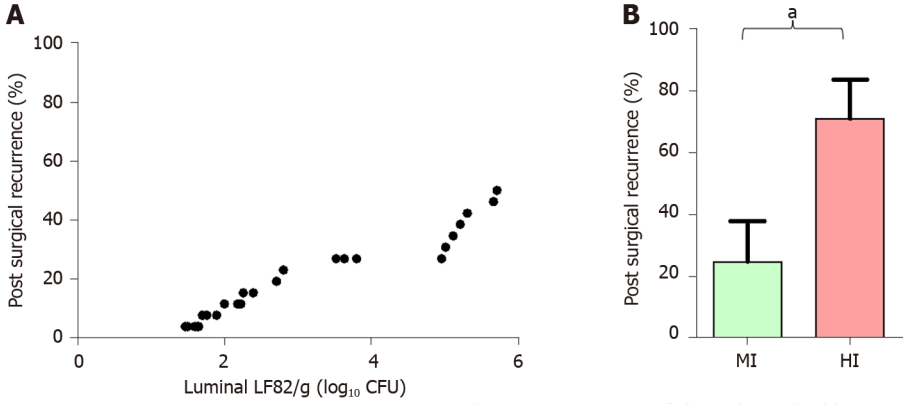
Figure 8 Evolution of the levels of luminal adherent-invasive *Escherichia coli* LF82 after surgery. A: Weekly evaluation of the luminal LF82 Levels after surgery in HLA-B27 transgenic (Tg) rats and wild-type (nTg) rats receiving adherent-invasive *Escherichia coli* strain LF82 alone or *Saccharomyces cerevisiae* CNCM I-3856 and LF82; B: Global persistence of viable luminal LF82 after surgery and during the last 5 wk of the study in Tg rats receiving LF82 alone or CNCM I-3856 and LF82. °P < 0.001. CFU: Colony-forming unit; log10: Decimal logarithm.

ileal lesions of CD and particularly in endoscopic POR occurring 6 mo after CD-related ileocolonic resection[12]. In the present study, we show that the probiotic *S. cerevisiae* CNCM I–3856 prevents LF82-induced POR occurring 6 wk after ICR in susceptible HLA-B27 Tg rats. In our model, oral administration of the LF82 AIEC strain induced POR in 85% of HLA-B27 Tg rats raised in a controlled pathogen-free facility. The lesions developed in a concentration-dependent manner to the amount of adherent LF82; moreover, they shared many similarities with CD lesions, including erosions and ulcers that could lead to stenosis, transparietal neutrophil infiltration, and a shift in cytokine profiles toward the IL-23/IL-17 axis. The goal of the postoperative management of CD is to identify patients at highest risk of recurrence to begin prophylactic treatment with biotherapies[8]. In our study, a high fecal concentration



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Figure 9 Correlation between anastomotic macroscopic scores and adherent and luminal adherent-invasive *Escherichia coli* LF82 levels. A: Adherent levels of adherent-invasive *Escherichia coli* strain LF82 at sacrifice [week (W) 18] were correlated with anastomotic macroscopic scores at sacrifice in paired transgenic (Tg) animals receiving LF82 alone or in combination with *Saccharomyces cerevisiae* CNCM I-3856; B: At surgery (W12), the levels of adherent LF82 were correlated with luminal LF82 Levels in paired Tg animals receiving LF82 alone; C: At W18, the levels of adherent LF82 were correlated with luminal LF82 Levels in paired Tg animals receiving LF82 alone. $^{a}P < 0.05$, $^{b}P < 0.01$. CFU: Colony-forming unit; log10: Decimal logarithm.



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Figure 10 Prognostic value of luminal adherent-invasive *Escherichia coli* LF82 levels in postoperative recurrence. A: Correlation between luminal adherent-invasive *Escherichia coli* strain LF82 Levels at week 14 and the risk of postoperative (POR) recurrence at W18 in transgenic (Tg) animals receiving LF82 alone or *Saccharomyces cerevisiae* (*S. cerevisiae*) CNCM I-3856 and LF82; B: Higher frequency of POR in highly infected (HI) Tg animals receiving LF82 alone or *S. cerevisiae* CNCM I-3856 and LF82 as defined by a cutoff value of 2.262 Log_{10} CFUs (colony-forming units) of luminal LF82 per gram of stool at W14 in comparison with mildly infected (MI) Tg rats (71.4% *vs* 25%, $P = 0.02$). $^{a}P < 0.05$. CFU: Colony-forming unit; log10: Decimal logarithm.

of LF82 had a 70% positive predictive value for POR occurring 4 wk later. The utility of this noninvasive diagnostic biomarker for predicting POR should be considered in future clinical studies evaluating the postoperative management of CD patients.

S. cerevisiae CNCM I-3856[22] is a probiotic yeast that has already been evaluated in large-scale clinical studies showing the safety and efficacy of this strain for abdominal pain management in patients with irritable bowel syndrome[22,32-34]. In the present study, daily oral administration of *S. cerevisiae* CNCM I-3856 at 10^{9} CFU/d was perfectly tolerated and reduced the severity and frequency of POR by more than 60% in HLA-B27 Tg rats. Moreover, an absence of LF82-induced POR without any macroscopic lesions was observed in 40% of transgenic animals treated preventively with *S. cerevisiae* CNCM I-3856. To our knowledge, this is the first time that a probiotic treatment showed such efficacy in preventing POR in a rodent preclinical model of POR of CD.

Different mechanisms of action may be involved in the therapeutic preventive effect of *S. cerevisiae* CNCM I-3856 against POR. Specific fractions of β6-glucan and α4-glucan expressed by *S. cerevisiae* CNCM I-3856 represent the strongest anti-adhesive yeast cell wall components against AIEC adhesion [24,35]. In our study, prevention of LF82-induced POR by *S. cerevisiae* CNCM I-3856 was associated with a significant decrease in adherent LF82 in the intestinal mucosa of animals together with a decrease in the persistence of luminal LF82, demonstrating the ability of *S. cerevisiae* CNCM I-3856 to decolonize AIEC from the gut of rats. Additional preclinical studies will be performed in our model using specific soluble glucan fractions of *S. cerevisiae* CNCM I-3856 to avoid the constraints of a live probiotic and to optimize the therapeutic efficacy. Another possible mechanism by which *S. cerevisiae* CNCM I-3856

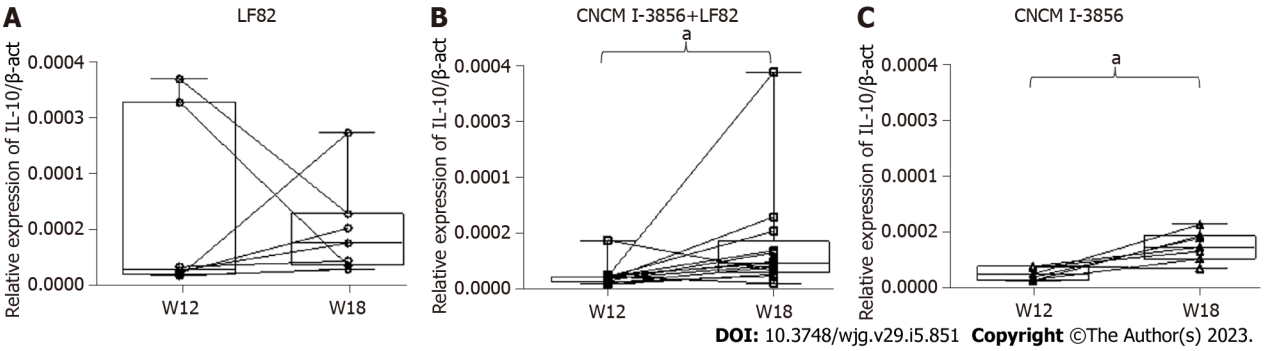


Figure 11 Interleukin-10 mRNA expression in the anastomotic mucosa. A: Interleukin (IL)-10 mRNA expression between surgery (week (W) 12) and sacrifice (W18) in paired Tg rats receiving adherent-invasive *Escherichia coli* strain LF82 alone; B: IL-10 mRNA expression between W12 and W18 in paired Tg rats receiving *Saccharomyces cerevisiae* (*S. cerevisiae*) CNCM I-3856 and LF82; C: IL-10 mRNA expression between W12 and W18 in paired Tg rats receiving *S. cerevisiae* CNCM I-3856 alone. $^{a}P < 0.05$. β-act: β-actin.

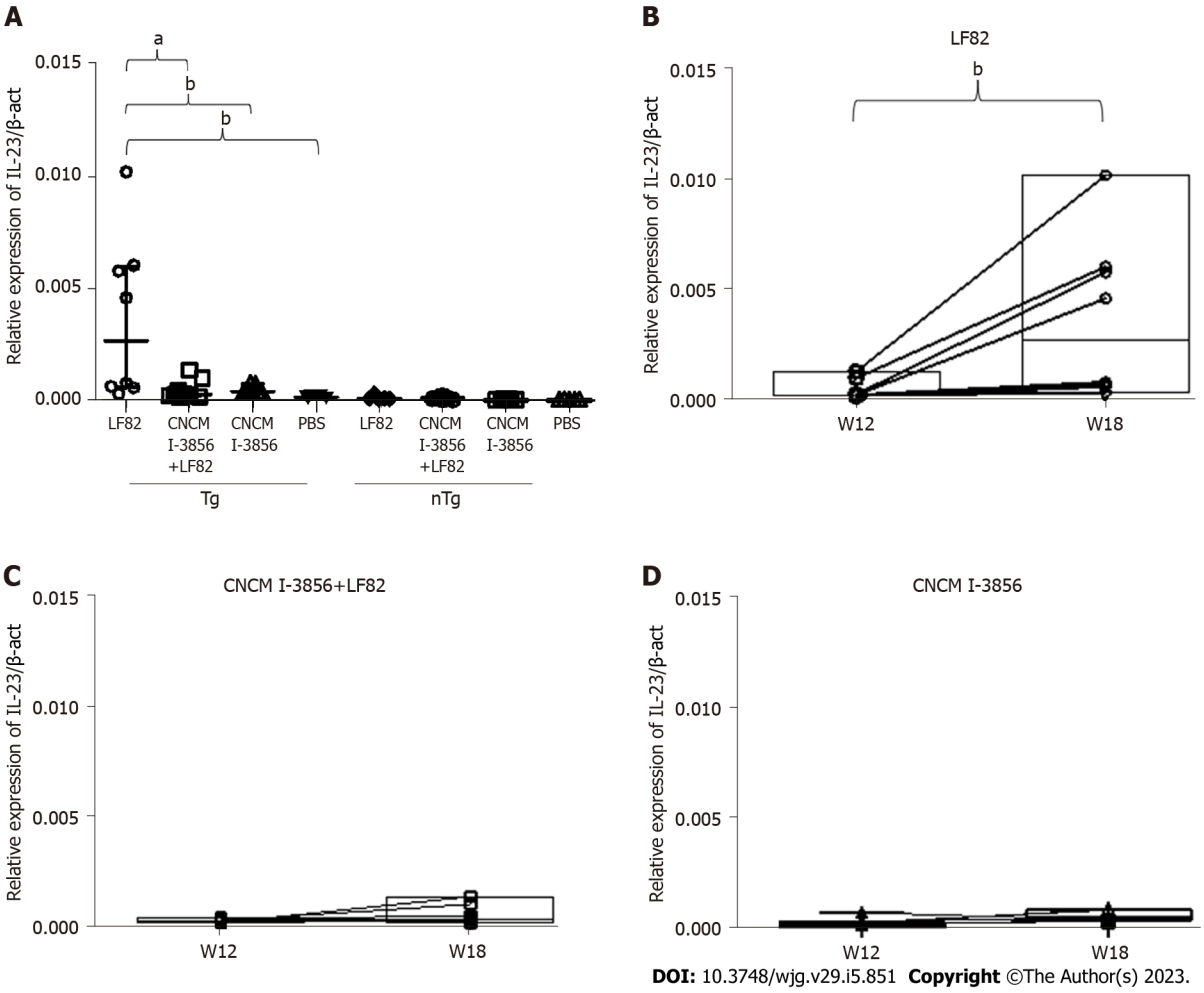
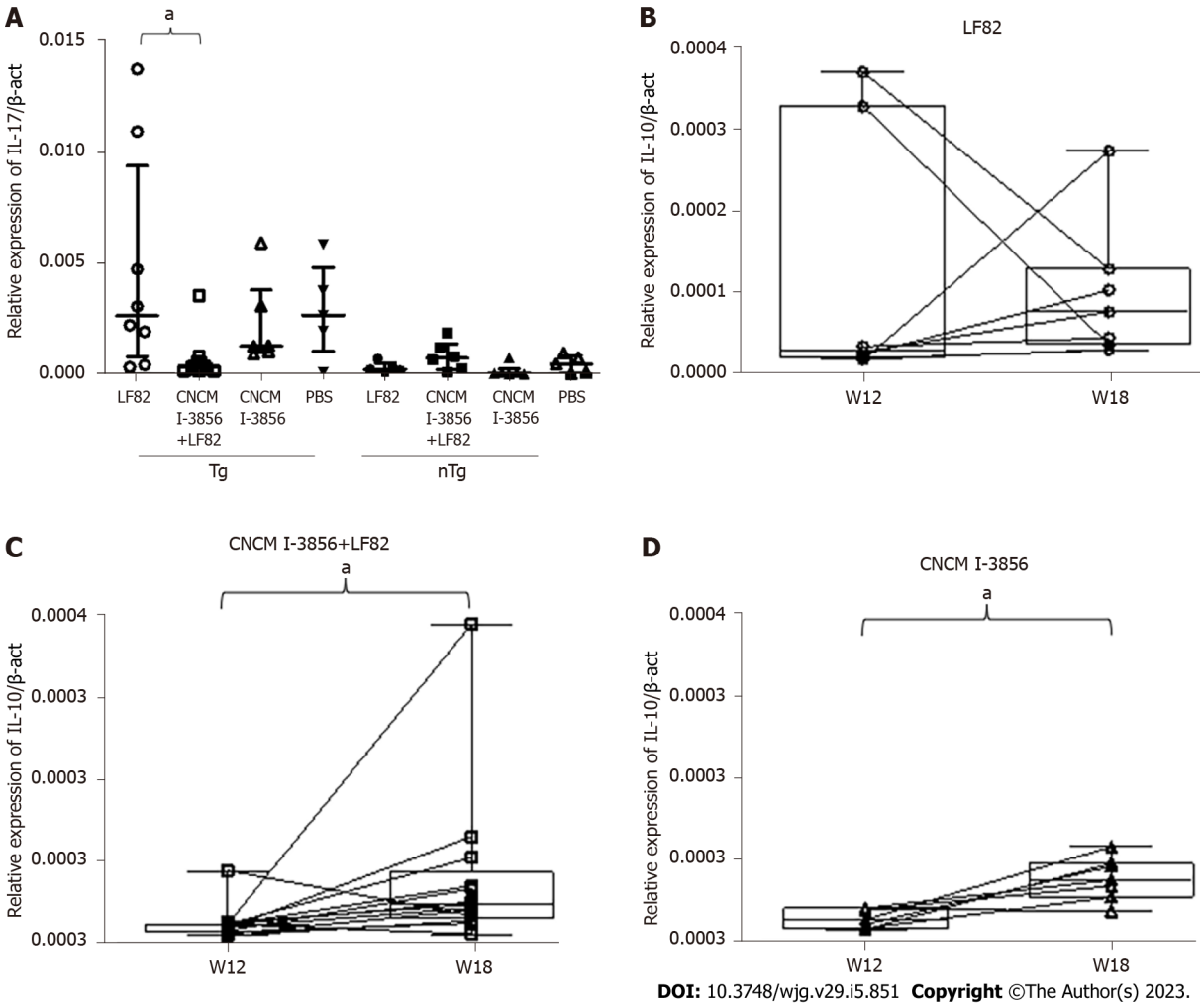


Figure 12 Interleukin-23 mRNA expression in the anastomotic mucosa. A: Expression of interleukin (IL)-23 mRNA in the perianastomotic mucosa in all transgenic (Tg) and nontransgenic (nTg) groups at sacrifice; B: IL-23 mRNA expression between surgery [week (W) 12] and sacrifice (W18) in paired Tg rats receiving adherent-invasive *Escherichia coli* strain LF82 alone; C: IL-23 mRNA expression between W12 and W18 in paired Tg rats receiving coadministration of *Saccharomyces cerevisiae* (*S. cerevisiae)* CNCM I-3856 and LF82; D: IL-23 mRNA expression between W12 and W18 in paired Tg rats receiving *S. cerevisiae* CNCM I-3856 alone. $^{a}P < 0.05$, $^{b}P < 0.01$. β-act: β-actin; PBS: Phosphate buffered saline.

prevents POR resides in its immunomodulatory and anti-inflammatory capacities[36]. We observed that the administration of *S. cerevisiae* CNCM I-3856 significantly increased IL-10 production in the intestine of rats and restored the local upregulation of IL-17 and IL-23 associated with LF82-induced POR in transgenic animals. The capacity of *S. cerevisiae* to induce IL-10 production has already been highlighted *in vitro* in bone-marrow dendritic cells and in porcine jejunal epithelial cells[36,37]. In the gut, IL-10 is



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Figure 13 Interleukin-17 mRNA expression in the anastomotic mucosa. A: Expression of interleukin (IL)-17 mRNA in the perianastomotic mucosa in all transgenic (Tg) and nontransgenic (nTg) groups at sacrifice; B: IL-17 mRNA expression between surgery [week (W) 12] and sacrifice (W18) in paired Tg rats receiving adherent-invasive *Escherichia coli* strain LF82 alone; C: IL-17 mRNA expression between W12 and W18 in paired Tg rats receiving coadministration of *Saccharomyces cerevisiae* (*S. cerevisiae*) CNCM I-3856 and LF82; D: IL-17 mRNA expression between W12 and W18 in paired Tg rats receiving *S. cerevisiae* CNCM I-3856 alone. $^{a}P < 0.05$. β-act: β-actin; PBS: Phosphate buffered saline.

produced by leukocytes and intestinal epithelial cells and plays important roles in maintaining gut homeostasis and harmonizing the interaction between host immunity and luminal microorganisms[38]. In a previous study of 79 patients with CD undergoing a first ileocolectomy and ileocolonic anastomosis, we reported that a low ileal IL-10 mRNA concentration was predictive of endoscopic recurrence occurring 3 mo later[39]. Thus, the ability of *S. cerevisiae* CNCM I-3856 to induce the intestinal production of IL-10 could be a key factor in preventing POR in our model.

CONCLUSION

In conclusion, our results identified *S. cerevisiae* CNCM I-3856 as a new and original candidate for the prevention of POR in selected AIEC-infected CD patients. In a reliable model of ICR in HLA-B27 Tg rats mimicking POR of CD, *S. cerevisiae* CNCM I-3856 was found to prevent macroscopic and histologic POR through a pathobiont AIEC-targeted mechanism and through its ability to induce intestinal IL-10 production. Given that the majority of patients with CD wish to have safe, natural, nonchemotherapeutic treatment, the *S. cerevisiae* CNCM I-3856 probiotic, which is already an alternative solution for the management of patients with irritable bowel syndrome because of its ability to alleviate abdominal pain and to improve quality of life, should represent a promising therapeutic solution in the management of postoperative CD.

ARTICLE HIGHLIGHTS

Research background

The presence of adherent-invasive *Escherichia coli* (AIEC) in intestinal flora is associated with postoperative recurrence (POR) of crohn's disease (CD). *Saccharomyces cerevisiae* (*S. cerevisiae*) CNCM I-3856 is a safe and effective probiotic yeast that has already been evaluated in randomized placebo-controlled studies in patients with irritable bowel syndrome. Preclinical studies demonstrate the capacity of *S. cerevisiae* CNCM I-3856 to agglutinate invasive *Escherichia coli* strains and to prevent their adhesion to intestinal epithelial cells, favoring AIEC elimination from the gut of mice.

Research motivation

To demonstrate that *S. cerevisiae* CNCM I-3856 should be considered as a postoperative prophylactic medical therapy in CD patients harboring AIEC bacteria.

Research objectives

To evaluate the beneficial effect of *S. cerevisiae* CNCM I-3856 and its mechanisms of action in preventing AIEC-induced POR in an HLA-B27 transgenic (TgB27) rat model of CD.

Research methods

TgB27 and control rats underwent an ileocecal resection at the 12th wk of life and sacrificed 6 wk later to assess POR using macroscopic and histological scores and quantification of mucosal inflammatory/regulatory cytokines. Animals were challenged daily with an oral administration of AIEC and were treated orally with *S. cerevisiae* CNCM I-3856 (10^9 colony forming units/day). Luminal and adherent AIEC were regularly quantified throughout the duration of the study.

Research results

Eighty-seven percent of TgB27 rats developed POR characterized by anastomotic macroscopic ulcerations, transparietal neutrophil infiltration and a shift in the cytokine profile toward the interleukin (IL)-17/IL-23 axis. Oral administration of *S. cerevisiae* CNCM I-3856 reduced this POR by more than 60%, increased AIEC elimination from the gut, induced intestinal IL-10 production and restored the local upregulation of IL-17/IL-23. A high concentration of AIEC quantified in the stool of rats after surgery had a 70% positive predictive value for POR occurring 4 wk later.

Research conclusions

Ileocecal resection in TgB27 rats is a novel, useful, reliable model mimicking POR of CD and aided the discovery of new therapeutic targets. Oral administration of *S. cerevisiae* CNCM I-3856 safely prevented POR of CD through AIEC decolonization and immunomodulatory/anti-inflammatory capacities.

Research perspectives

The probiotic *S. cerevisiae* CNCM I-3856, which is already an alternative solution for the management of patients with irritable bowel syndrome to improve abdominal pain and quality of life, should represent a promising prophylactic natural nonchemotherapeutic solution in the management of postoperative CD. Monitoring AIEC levels in stool after surgery for CD should be considered as a companion test to identify patients at high risk of POR and to monitor treatment response.

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FOOTNOTES

Author contributions: Desreumaux P, Dubuquoy C and Valibouze C designed the study; Valibouze C, Dubuquoy C, M'Ba L, Schneider L and Neut C acquired the data; Genin M supervised the statistical analysis; all authors interpreted the data; Valibouze C and Desreumaux P drafted the article; All authors critically reviewed the manuscript and approved the final version for submission.

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Conflict-of-interest statement: Mourey F is an employee of Lesaffre. Desreumaux P reports personal fees from Abbvie, personal fees from Abbott, personal fees from Amgen, personal fees from Biocodex, personal fees from Biofortis, personal fees from Biogen, personal fees from Biokuris, personal fees from Ferring, personal fees from Fresenius, personal fees from Janssen, personal fees from Kitozyme, personal fees from Lesaffre, personal fees from MSD, personal fees from Norgine, personal fees from Pfizer, personal fees from Sandoz, personal fees from Shire, personal fees from Takeda, personal fees from Tillotts, and personal fees from UCB, outside of the submitted work. In addition, Dr. Desreumaux has a patent (WO2009103884) issued. All other authors have nothing to disclose.

Data sharing statement: The dataset is available from the corresponding author at caroline.valibouze@chu-lille.fr.

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Country/Territory of origin: France

ORCID number: Caroline Valibouze 0000-0002-2198-1392; Silvia Speca 0000-0001-8494-3329; Caroline Dubuquoy 0000-0003-1584-3601; Florian Mourey 0000-0002-0826-9632; Lena M'Ba 0000-0002-9564-9472; Lucil Schneider 0000-0001-7486-5099; Marie Titecat 0000-0002-5860-0936; Benoît Foligné 0000-0001-9263-9706; Michaël Genin 0000-0002-9098-7528; Christel Neut 0000-0002-2036-6152; Philippe Zerbib 0000-0002-6466-0716; Pierre Desreumaux 0000-0002-6127-5281.

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ORIGINAL ARTICLE

Basic Study

Impact of endothelial nitric oxide synthase activation on accelerated liver regeneration in a rat ALPPS model

Hitoshi Masuo, Akira Shimizu, Hiroaki Motoyama, Koji Kubota, Tsuyoshi Notake, Takahiro Yoshizawa, Kiyotaka Hosoda, Koya Yasukawa, Akira Kobayashi, Yuji Soejima

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Hitoshi Masuo, Akira Shimizu, Hiroaki Motoyama, Koji Kubota, Tsuyoshi Notake, Takahiro Yoshizawa, Kiyotaka Hosoda, Koya Yasukawa, Akira Kobayashi, Yuji Soejima, Division of Gastroenterological, Hepato-Biliary-Pancreatic, Transplantation and Pediatric Surgery, Department of Surgery, Shinshu University School of Medicine, Matsumoto 390-8621, Japan

Corresponding author: Akira Shimizu, MD, PhD, Associate Professor, Division of Gastroenterological, Hepato-Biliary-Pancreatic, Transplantation and Pediatric Surgery, Department of Surgery, Shinshu University School of Medicine, 3-1-1, Asahi, Matsumoto 390-8621, Japan. ashimizu@shinshu-u.ac.jp

Abstract

BACKGROUND

Although the associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) induces more rapid liver regeneration than portal vein embolization, the mechanism remains unclear.

AIM

To assess the influence of inflammatory cytokines and endothelial nitric oxide synthase (eNOS) activation on liver regeneration in ALPPS.

METHODS

The future liver remnant/body weight (FLR/BW) ratio, hepatocyte proliferation, inflammatory cytokine expression, and activation of the Akt-eNOS pathway were evaluated in rat ALPPS and portal vein ligation (PVL) models. Hepatocyte proliferation was assessed based on Ki-67 expression, which was confirmed using immunohistochemistry. The serum concentrations of inflammatory cytokines were measured using enzyme linked immune-solvent assays. The Akt-eNOS pathway was assessed using western blotting. To explore the role of inflammatory cytokines and NO, Kupffer cell inhibitor gadolinium chloride ($GdCl_3$), NOS inhibitor N-nitro-arginine methyl ester (L-NAME), and NO enhancer molsidomine were administered intraperitoneally.

RESULTS

The ALPPS group showed significant FLR regeneration (FLR/BW: 1.60% ± 0.08%, $P < 0.05$) compared with that observed in the PVL group (1.33% ± 0.11%) 48 h after surgery. In the ALPPS group, serum interleukin-6 expression was suppre-

ssed using $GdCl_3$ to the same extent as that in the PVL group. However, the FLR/BW ratio and Ki-67 labeling index were significantly higher in the ALPPS group administered $GdCl_3$ (1.72% ± 0.19%, $P < 0.05$; 22.25% ± 1.30%, $P < 0.05$) than in the PVL group (1.33% ± 0.11% and 12.78% ± 1.55%, respectively). Phospho-Akt Ser^{473} and phospho-eNOS Ser^{1177} levels were enhanced in the ALPPS group compared with those in the PVL group. There was no difference between the ALPPS group treated with L-NAME and the PVL group in the FLR/BW ratio and Ki-67 labeling index. In the PVL group treated with molsidomine, the FLR/BW ratio and Ki-67 labeling index increased to the same level as in the ALPPS group.

CONCLUSION

Early induction of inflammatory cytokines may not be pivotal for accelerated FLR regeneration after ALPPS, whereas Akt-eNOS pathway activation may contribute to accelerated regeneration of the FLR.

Key Words: Hepatectomy; Nitric oxide; Liver regeneration; Cytokines; NG-Nitroarginine methyl ester; Molsidomine

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Core Tip: In extended hepatectomy for hepatobiliary tumors, adequate future liver remnant (FLR) is essential to prevent postoperative liver failure. Portal vein embolization (PVE) and associated liver partition and portal vein ligation for staged hepatectomy (ALPPS) are performed to increase the FLR. Although ALPPS induces more rapid liver regeneration than PVE, the mechanism remains unclear. In this study, we compared ALPPS with portal vein ligation (PVL) in a rat model and found that activation of the Akt-endothelial nitric oxide synthase pathway promotes liver regeneration. The combination of PVL and nitric oxide-producing agents may induce liver regeneration comparable to ALPPS in a non-invasive manner.

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INTRODUCTION

Hepatectomy is the most curative treatment for hepatobiliary carcinoma[1,2]. Extended hepatectomy is occasionally performed to achieve R0 surgical margins. However, postoperative liver failure may occur in these cases because of an inadequate volume of the future liver remnant (FLR)[3,4]. To resolve this issue, portal vein embolization (PVE) is widely performed before major hepatectomy to obtain a sufficient FLR volume[5,6]. Although PVE results in a 10%-45% increase in FLR, it requires a waiting period of 2-8 wk[6-8]. Hepatectomy cannot be performed in some cases because of tumor progression, inadequate volume increase, or both in the FLR, even after PVE. Therefore, the resection rate after PVE has been reported as only 70%[7,8]. Furthermore, it has been reported that hepatocellular carcinoma (HCC) is nourished by abnormal vessels in the hepatic artery (HA). Thus, PVE may reduce blood flow in the portal vein and increase blood flow in the HA of the liver to be resected, which may result in rapid progression of HCC[9]. As described above, PVE has limited indications and therapeutic effects. Therefore, the development of new surgical or therapeutic methods is desired to promote further liver regeneration in the short term.

As an alternative to PVE, associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) was reported in 2012[6]. This method enables the FLR to increase by 70%-80% within 10 d[6]. ALPPS promotes a much faster increase of FLR than PVE[6,9], but the mechanism of this rapid liver regeneration remains unclear. Although increases in inflammatory cytokines, such as interleukin-6 (IL-6), which is an inducer in the early stage of liver regeneration, have been reported as a cause of rapid liver regeneration[10-13], it remains controversial[14]. However, in previous studies on the mechanism of liver regeneration after liver resection and portal vein ligation (PVL), shear stress caused by blood viscosity, blood flow velocity, and endothelial nitric oxide synthase (eNOS) activation, followed by NO induction, has been reported to promote liver regeneration[15,16]. This study aimed to explore the mechanism of promoting liver regeneration in ALPPS and investigate the involvement of inflammatory

cytokines and eNOS activation using PVL and ALPPS rat models.

MATERIALS AND METHODS

Animals

Eight-week-old male Wistar rats (CLEA Japan, Kanagawa, Japan) weighing 230-300 g were used in this study. The animals were housed in wood-chip-bedded cages in an air-conditioned room (24 ± 1 °C) with a 12 h light/dark cycle under specific pathogen free condition. There were no diet restrictions. Based on national and institutional regulations and guidelines, all procedures for animal experiments were reviewed by the Committee for Animal Experiments and approved by the President of Shinshu University (Approval numbers 270018 and 019067).

Surgical procedures and study design

Rats were divided into two groups, PVL and ALPPS, and examined 72 h after surgery. A midline laparotomy was performed under isoflurane-induced anesthesia. In the PVL model, the portal vein branches to the caudate lobe, left lobe, left side of the median lobe, and right lobes were ligated with 7-0 silk (Figure 1A). In the ALPPS model, in addition to PVL, liver parenchymal transection between the right lobe and the left side of the middle lobe was performed based on the gross morphology and demarcation line after PVL. The Glisson flowing into the left side of the median lobe was ligated with 7-0 nylon (Figure 1B). Little bleeding occurred during the liver parenchymal transection because the parenchyma on either side of the dissection line was ligated with 6-0 Prolene before parenchymal transection to control intraoperative bleeding. The abdomen was then closed in layers.

The rats were sacrificed to collect blood samples and liver tissue from the right side of the median lobe (RML) at 1, 4, 6, 24, 48, and 72 h after surgery (n = 5 for each group per time point). Blood samples were collected from the inferior vena cava at the time of liver removal and centrifuged at 2600 × g for 5 min. The serum was stored at -80 °C. Liver tissue samples were frozen in liquid nitrogen and stored at -80 °C. The remaining liver tissue was fixed with 4% paraformaldehyde.

The weight of the FLR, that is, the RML, and body weight (BW) were measured before surgery and at 24, 48, and 72 h after surgery. The BW (FLR/BW) ratio (%) was used as the liver regeneration index. In western blotting analysis and volumetric blood flow analysis, the PVL and ALPPS groups were compared based on the control group, in which only open and closed abdomens were performed.

ELISAs of serum inflammatory cytokines and hepatocyte growth factor

Serum concentrations of IL-6, tumor necrosis factor-α (TNF-α), and hepatocyte growth factor (HGF) were measured at 1, 4, 6, and 24 h after surgery using ELISA kits (R&D Systems, Minneapolis, MN, United States). IL-6 concentration in the RML tissue was also quantified 1 h after surgery.

Immunohistochemistry

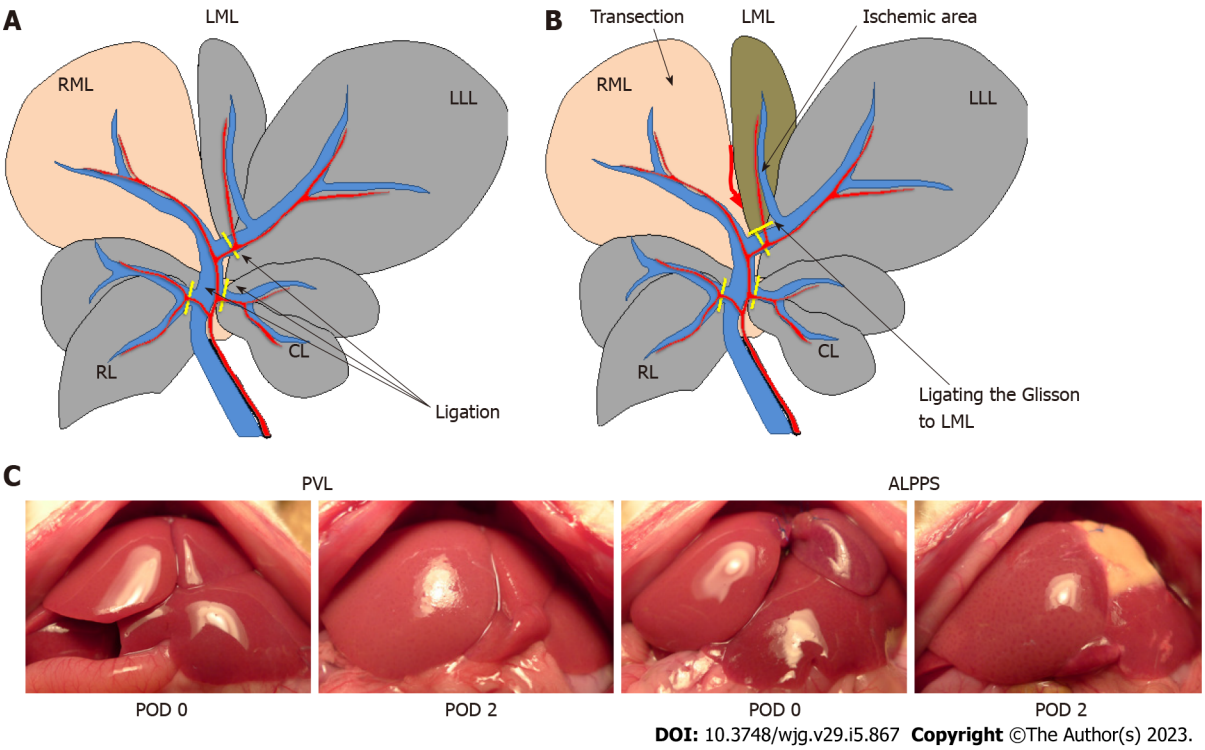
The liver tissues were fixed with paraformaldehyde and embedded in paraffin. After deparaffinization, antigen retrieval, and quenching of endogenous peroxidases, the sections were incubated overnight at 4 °C with a mouse monoclonal anti-Ki-67 antibody (1:200 dilution; Dako, Glostrup, Denmark; 1:200 dilution, Abcam, Cambridge, United Kingdom), followed by incubation for 30 min at room temperature with a peroxidase-labeled anti-mouse antibody (Histofine Simplestain Max PO; Nichirei). The sections were immersed in diaminobenzidine solution for visualization and counterstained with hematoxylin. To evaluate hepatocyte proliferation 48 h after surgery, the average percentage of Ki-67-positive cells to total hepatocytes in three random high-power fields was used as the Ki-67 labeling index.

Kupffer cell inhibition in the ALPPS model

To explore the role of inflammatory cytokines in liver regeneration, the Kupffer cell inhibitor gadolinium chloride ($GdCl_3$; Sigma-Aldrich, St. Louis, MO, United States) was used. Another set of animals was used for the Kupffer cell inhibition experiments. We prepared an ALPPS model for $GdCl_3$ administration (n = 3). $GdCl_3$ (10 mg/kg) was administered intraperitoneally 24 h before surgery. In the control group, physiological saline was administered. All rats were sacrificed 48 h after surgery to obtain liver samples.

NOS inhibition in the ALPPS model and NO enhancement in the PVL model

To explore the role of NO in liver regeneration, the NOS inhibitor NG-nitro-arginine methyl ester (L-NAME; Sigma-Aldrich) and the NO enhancer molsidomine (Cayman Chemical, MI, United States) were used. Another set of animals was used for the NOS inhibition and NO enhancement experiments. We prepared the ALPPS model for L-NAME administration, the PVL model for molsidomine administration, and the corresponding control PVL and ALPPS models (n = 5 for each group). L-NAME (100 mg/kg) or molsidomine (10 mg/kg) was administered intraperitoneally 24 h before and during surgery. In each control group, physiological saline was administered. All rats were sacrificed 24, 48, and 72 h



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Figure 1 Schema of experimental models. A: Portal vein ligation (PVL) group. Portal vein branches were ligated, other than the right median lobe; B: Associating liver partition and PVL for staged hepatectomy group. In addition to ligating the portal vein as performed in the PVL group, the median lobe was transected, and the left Glisson was ligated; C: Macroscopic findings after operations in each group. ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy; PVL: Portal vein ligation; RML: Right median lobe; LML: Left median lobe; LLL: Left lateral lobe; RL: Right lobe; CL: Caudate lobe; POD: Postoperative day.

after surgery to obtain liver samples.

Western blot analysis

The RML tissue proteins were collected at 1, 4, and 6 h after surgery using radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology, Inc., CA, United States). The protein concentration was measured using the bicinchoninic acid assay method. Samples of 10 μg proteins from FLRs of PVL and ALPPS models were separated on 4%-12% NuPAGE Gels and transferred onto nitrocellulose membranes. After blocking with 5% dry skim milk for 1 h, the membranes were incubated with primary antibodies overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h. The blots were developed with ECL Select western blotting Detection Reagent (Amersham, GE Healthcare Life Sciences, Chicago, IL, United States) and photographed using a Molecular Imager ChemiDoc XRS device (Bio-Rad Laboratories, Inc., Hercules, CA, United States). The density of the bands in the immunoblots was analyzed using Image Lab Software (Bio-Rad Laboratories, Inc.). The results are expressed as a percentage of the β-actin internal control. The anti-human antibodies used were rabbit monoclonal antibodies against p-Akt (Ser 473) (cat. no. 4060), p-eNOS (Ser1177) (Cat. no. 9570), p-eNOS (Thr495) (Cat. no. 9574), total eNOS (Cat. no. 32027) (Cell Signaling Technology, Inc., Danvers, MA, United States), and mouse monoclonal antibody against β-actin (Cat. no. A5441; Sigma-Aldrich). Anti-β-actin antibody was used at a 1:3000 dilution, and the other antibodies were used at a 1:1000 dilution.

Volumetric blood flow analysis

Before the estimation of volumetric blood flow in the HA and PV of the FLR, blood velocity and vascular diameter (r) were measured using ultrasonography (Vevo2100, Primetech, Tokyo, Japan). Volumetric blood flow was estimated from the blood velocity and vascular cross-sectional area (πr^2) (volumetric blood flow = blood velocity × πr^2) in mm^3 per second.

Statistical analysis

The collected data were evaluated statistically using the JMP software, version 13.2 (SAS Institute, Cary, NC, United States). Data are expressed as mean ± SD. Statistical analysis was performed using an unpaired student's *t*-test. Statistical significance was defined as $P < 0.05$.

RESULTS

Differences in liver regeneration in PVL and ALPPS models

The FLR/BW ratio increased over time in both groups. At 48 h after surgery, the FLR/BW ratio in the ALPPS group was significantly higher (1.60% ± 0.08%, $P < 0.05$) than that in the PVL group (1.33% ± 0.11%) (Figures 1C and 2A). However, no significant difference was observed between the two groups at 24 and 72 h after surgery. The Ki-67 labeling index of the RML at 48 h after surgery was significantly increased in the ALPPS group (22.1% ± 4.01%, $P < 0.05$) compared with that in the PVL group (12.8% ± 1.73%), which was consistent with the FLR/BW ratio (Figures 2B and 2C).

Association between serum inflammatory cytokines and liver regeneration

The serum concentrations of IL-6, TNF-α, and HGF in RML were measured at 1, 4, 6, and 24 h after surgery. Serum IL-6 and TNF-α levels increased in both groups after surgery compared with the levels before surgery. However, no difference was found in the two groups at 1, 4, and 6 h after surgery. At 24 h after surgery, IL-6 and TNF-α concentrations were significantly higher in the ALPPS group (25.91 ± 6.05 pg/mL, $P < 0.05$ and 1.52 ± 0.68 pg/mL, $P < 0.05$) compared with that in the PVL group (4.11 ± 3.99 pg/mL and 0.54 ± 0.38 pg/mL). Serum HGF concentration at 1 h after surgery was significantly higher in the ALPPS group (68.86 ± 4.89 ng/mL, $P < 0.05$) compared with that in the PVL group (55.34 ± 9.97 ng/mL). However, no significant difference was observed in serum HGF concentration at 4, 6, and 24 h (Figures 3A-C).

Liver regeneration in the ALPPS model under the suppression of IL-6 using $GdCl_3$

To evaluate the effect of IL-6 on liver regeneration, ALPPS rats were administered $GdCl_3$, which suppressed the activation of Kupffer cells in the liver. In the $GdCl_3$-ALPPS group, the IL-6 concentrations in serum (40.3 ± 11.3 pg/mL, $P < 0.05$) and the RML tissue (3.27 ± 0.54 ng/TP 1 g, $P < 0.05$) 1 h after surgery were significantly decreased compared with the concentrations in the corresponding groups without administration of $GdCl_3$ (Figures 4A and 4B). However, there was no significant difference in the FLR/BW ratio or Ki-67 labeling index at 48 h after surgery in the ALPPS group with or without administration of $GdCl_3$ (Figures 4C and 4D).

Short-term postoperative liver regeneration induced by eNOS

Phosphorylation of Akt and eNOS in RML tissue at 1, 4, and 6 h after surgery was evaluated using western blotting (Figure 5A). Phospho-Akt Ser^{473} and phospho-eNOS Ser^{1177} levels increased in the ALPPS group compared with those in the PVL group. The quantitative measurement revealed that the phosphorylation levels of eNOS Ser^{1177} in the ALPPS group was significantly higher than that in the PVL group at 1 and 4 h after surgery. However, there was no significant difference at 6 h after surgery (Figure 5B).

L-NAME, an NOS inhibitor, was administered to rats to examine whether suppression of eNOS affected liver regeneration. The FLR/BW ratio and Ki-67 labeling index at 48 h after surgery in the L-NAME-ALPPS group were significantly lower than those in the ALPPS group without L-NAME administration and were comparable to those in the PVL group (Figures 6A and 6B).

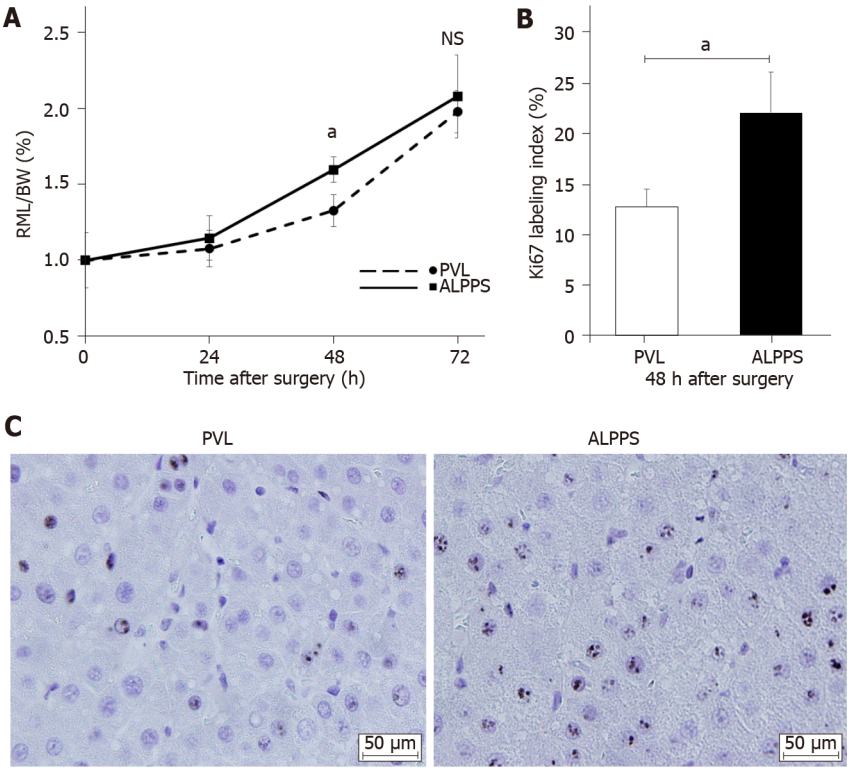
Additionally, molsidomine, which induces eNOS activation, was administered to the rats to examine whether eNOS activation affects liver regeneration. The FLR/BW ratio and Ki-67 labeling index at 48 h after surgery in the molsidomine-administered PVL (molsidomine-PVL) group were significantly higher than those in the PVL group without molsidomine administration and comparable with those in the ALPPS group (Figures 6C and 6D). However, there was no significant difference in the long-term FLR/BW ratio on a postoperative day 7 between the PVL, ALPPS, and molsidomine-administered PVL groups (data not shown).

Increased HA blood flow in the ALPPS model

PV flow in the PVL and ALPPS groups was significantly faster than that in the control group; however, there was no significant difference in PV flow between the PVL and ALPPS groups (180.1 ± 54.4, 216.6 ± 71.4 mm^3/s) (Figure 7A). HA flow in the PVL group was significantly slower than that in the control group without surgical intervention (1.73 ± 1.14 *vs* 3.66 ± 0.74 mm^3/s, $P < 0.05$), whereas that in the ALPPS group was significantly faster (11.32 ± 2.40 mm^3/s, $P < 0.05$) than that in control and PVL groups (Figure 7B). The total blood flow, that is, the sum of PV and HA, was not significantly different between the PVL and ALPPS groups (Figure 7C).

DISCUSSION

Hepatectomy is the most curative treatment for HCC and intraductal cholangiocarcinoma[1,2]. Additionally, major hepatectomy is the standard operative procedure for perihilar cholangiocarcinoma [17,18]. Extended hepatectomy may be required, depending on the location of the cancer. Postoperative



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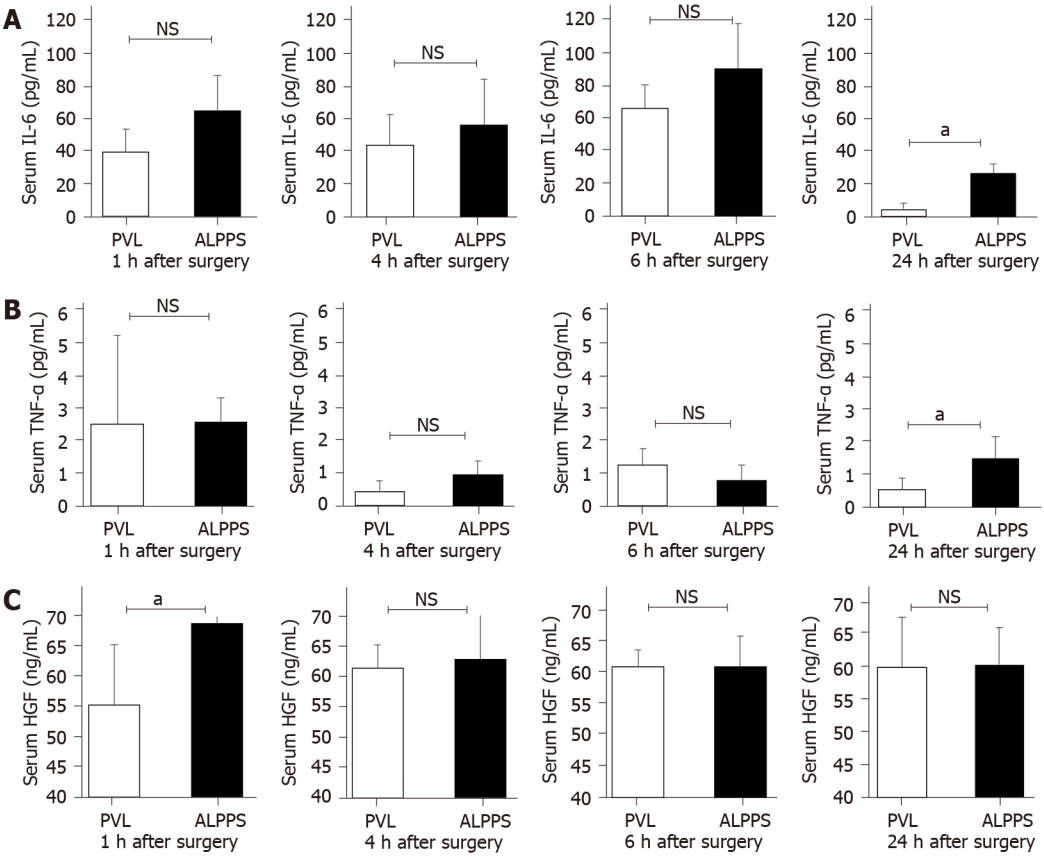
Figure 2 Changes in the right side of the median lobe weight to body weight ratio and Ki-67 index after surgery. A: Future liver remnant/body weight ratio up to 72 h after surgery; B: Immunohistochemistry of Ki-67 at 48 h after the operation; C: Ki-67 labeling index at 48 h after the surgery. Values are expressed as the mean ± SD; $n = 5$ for each group; $^{a}P < 0.05$; NS: Not significant; RML/BW: Right side of the median lobe weight/body weight; PVL: Portal vein ligation; ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy.

liver failure that results from insufficient residual liver volume is a fatal complication of hepatectomy. PVE and ALPPS were developed with the aim of pre-operative liver enlargement to avoid postoperative liver failure[5,6,18]. ALPPS leads to the rapid regeneration of FLR compared with PVE, although high mortality (90-d mortality of 9%) and morbidity (grade IIIb of 40% in the Clavien-Dindo classification) are limitations[19]. Elucidation of the mechanism of rapid liver regeneration after ALPPS may contribute to improving surgical outcomes for patients who undergo extended hepatectomy for hepatobiliary malignancies and to the development of novel alternative treatments that provide effective and safe regeneration of the FLR.

In this study, we obtained two crucial findings regarding the mechanism of liver regeneration in ALPPS. First, the induction of inflammatory cytokines, such as IL-6, might not be pivotal for the rapid regeneration of FLR after ALPPS in the early phase. Second, activation of the Akt-eNOS pathway may be an important factor in promoting liver regeneration after ALPPS.

The mechanism of liver regeneration has been studied in animal models of partial hepatectomy. The regeneration process is distinctive, complex, and well-coordinated and depends on the interactions of several signaling pathways, cytokines, and growth factors. Additionally, endocrine hormones, such as norepinephrine, growth hormone, insulin, and thyroid hormones, have been reported to influence these pathways and factors[20-22]. Since Schnitzbauer *et al*[6] reported ALPPS in 2012, there have been several reports to elucidate the major factors in liver regeneration of ALPPS, which promote rapid liver regeneration compared with PVL[10-13,23]. Activation of downstream signals, such as c-Jun N-terminal kinase-Indian hedgehog signaling from stellate cells by inflammatory cytokines[24], activation of the Janus kinase 2/signal transducer and activator of transcription 3 pathway *via* regenerating islet-derived 3α/3β, and hypoxia-induced stabilization of hypoxia-inducible factor-α subunits by hypoxia[14,25,26], have been reported as major factors. However, the mechanism of liver regeneration in ALPPS has not yet been completely elucidated.

Previous studies have reported that the peak of cell proliferation is 48 h after surgery, and inflammatory cytokines and their downstream signal enhancement cause liver regeneration in ALPPS[11,12,23]. Although the peak liver regeneration in this study was consistent with previous studies, the relationship between the early induction of inflammatory cytokines and liver regeneration was not consistent. In this study, serum concentrations of inflammatory cytokines, such as IL-6 and TNF-α, in the short term (1, 4, and 6 h) after surgery did not differ between the ALPPS and PVL groups. However, the ALPPS group showed a greater increase in FLR and a higher Ki-67 labeling index than in the PVL group. Additionally, suppression of inflammatory cytokines using $GdCl_3$ did not suppress liver

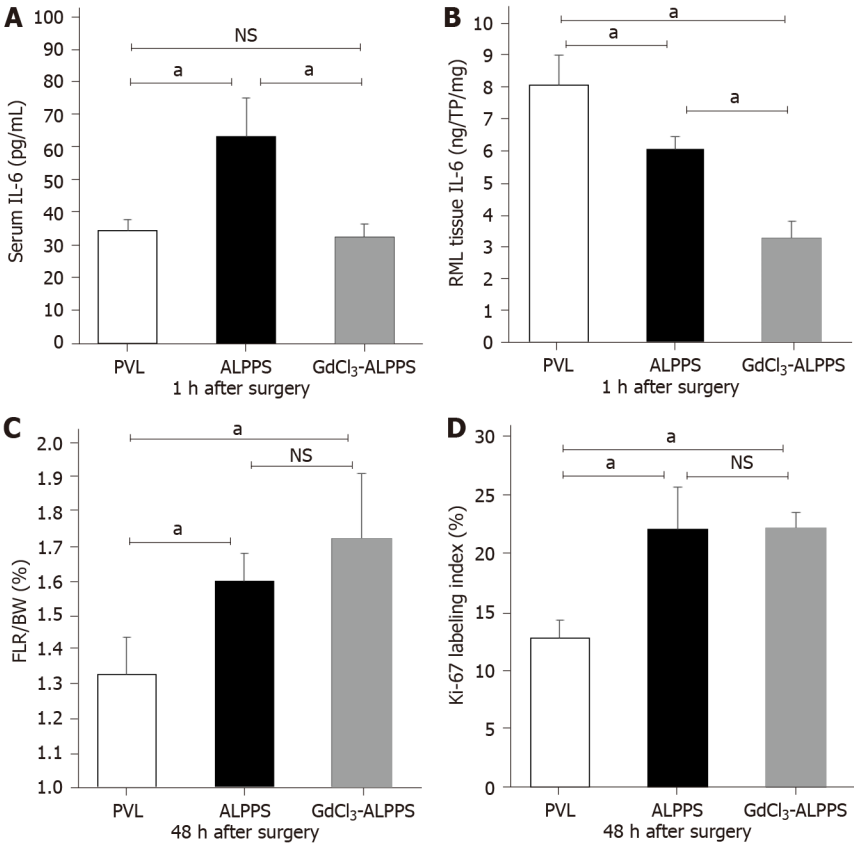


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Figure 3 Expression of inflammatory cytokines and hepatocyte growth factor in serum and right side of the median lobe tissue. A: Serum interleukin-6 concentrations at 1, 4, 6, and 24 h after surgery; B: Tumor necrosis factor-α concentrations at 1, 4, 6, and 24 h after surgery; C: Hepatocyte growth factor concentration at 1, 4, 6, and 24 h after surgery. Values are expressed as the mean ± SD; $n = 5$ for each group; $^{a}P < 0.05$; NS: Not significant; PVL: Portal vein ligation; ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy; IL: Interleukin; TNF: Tumor necrosis factor; HGF: Hepatocyte growth factor.

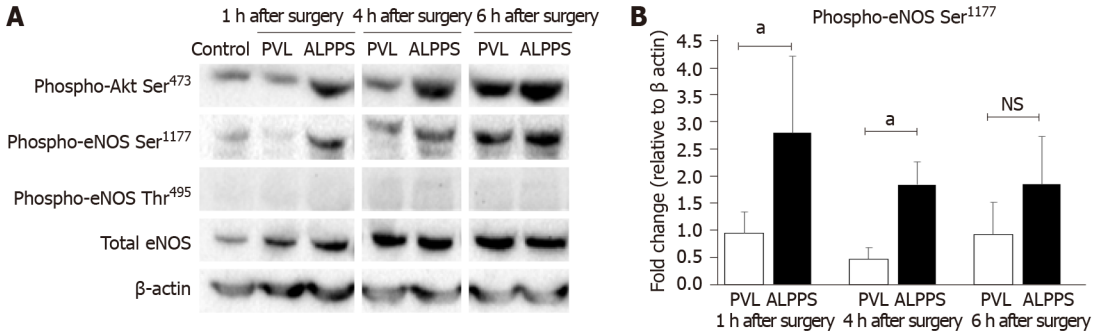
regeneration. These results suggest that the induction of inflammatory cytokines in the early phase after ALLPS is not necessarily a major factor in accelerating liver regeneration. The reason why no difference was observed in the expression of inflammatory cytokines may be the site of liver resection, setting of FLR, or differences in animal models. The timing of specimen collection may have influenced the results, as specimens collected 24 h after surgery had higher concentrations in the ALPPS group.

Activation of eNOS and NO induction have been reported to be a mechanism of liver regeneration other than inflammatory cytokines[15,16]. In this study, we focused on the effect of eNOS activation on liver regeneration after PVL and ALPPS. Evaluation of eNOS activation in the liver tissue showed that eNOS Ser^{1177} phosphorylation was significantly increased in the ALPPS model at 1 and 4 h after surgery. Thus, the FLR/BW ratio and Ki-67 labeling index in the ALPPS model were increased compared with those in the PVL model. Furthermore, the activation of Akt, which is upstream of eNOS, was observed, suggesting that the Akt-eNOS pathway contributes to the mechanism of liver regeneration in ALPPS. The administration of L-NAME, which suppresses NO, inhibits liver regeneration. The administration of molsidomine, which activates eNOS, promotes liver regeneration. Molsidomine is a nitrate drug used as a coronary vasodilator for the treatment of angina pectoris; its intermediate metabolite, SIN-1 (ionidamine chlorohydrate) produces NO[27]. When endothelial cells are stimulated by shear stress or vascular endothelial growth factor, phosphoinositide 3-kinase (PI3K) is activated and PIP3 is produced, which activates the PI3K-Akt pathway and activates downstream signals such as eNOS[28,29]. An increase in shear stress, which has been reported to cause NO production[30], is due to hemodynamic changes in the residual liver caused by hepatectomy, which is expected to affect liver regeneration in ALPPS. To evaluate the effect of increased shear stress on liver regeneration, we examined the blood flow exchange after PVL and ALPPS. Contrary to our expectations, there was no difference in PV or total blood flow, which might be associated with shear stress, between the PVL and ALPPS groups; however, HA flow in the ALPPS group was significantly higher than that in the PVL and control groups. Therefore, the difference in oxygenation of the FLR, rather than the shear stress between ALPPS and PVL, might be associated with the difference in liver regeneration. However, Schadde *et al*[25] reported that hypoxia due to reduced HA flow in the FLR promotes hepatic regeneration in patients who underwent ALPPS and in the rat ALPPS model. However, in their study, HA flow was evaluated



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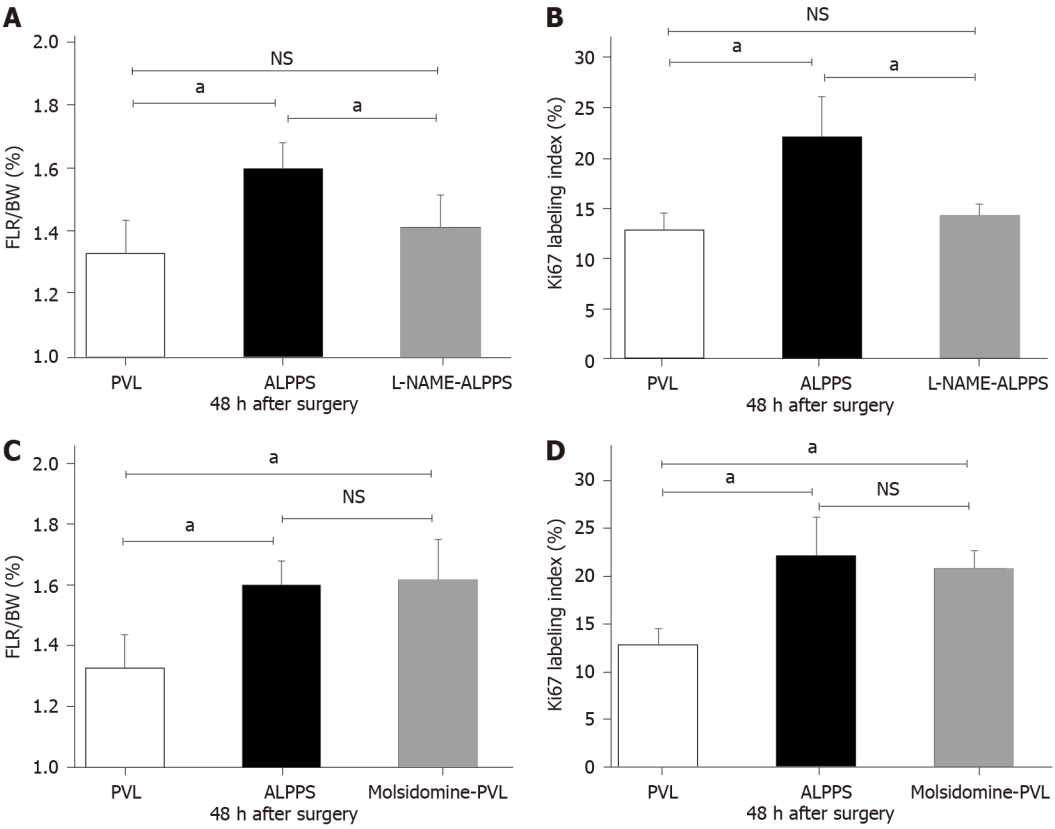
Figure 4 Interleukin-6 expression and liver regeneration in the gadolinium chloride model. A: The serum concentration of interleukin (IL)-6 at 1 h after surgery; B: IL-6 concentration in right side of the median lobe tissue at 1 h after surgery; C: Future liver remnant/body weight ratio at 48 h after surgery; D: Ki-67 labeling index. Values are expressed as mean ± SD; n = 3 or 5 for each group; $^{a}P < 0.05$; NS: Not significant; PVL: Portal vein ligation; ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy; IL: Interleukin; RML: Right median lobe; FLR/BW: Future liver remnant/body weight; $CdCl_3$: Gadolinium chloride.



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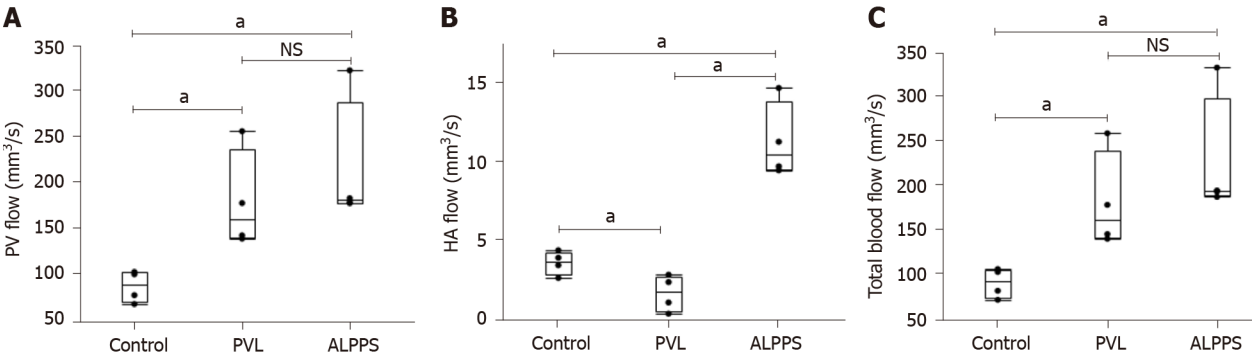
Figure 5 Western blotting of Akt-endothelial nitric oxide synthase pathway-related proteins. A: Western blotting was used to evaluate the expression of phosphorylated Akt and endothelial nitric oxide synthase (eNOS) in right side of the median lobe at 1, 4, and 6 h after surgery in portal vein ligation (PVL) and associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) groups; B: Comparison of the expression of P-Akt Ser^{473} and P-eNOS Ser^{1177} in PVL and ALPPS groups (quantification of western blots, n = 5 for each group). Values are expressed as the mean ± SD; n = 5 for each group; $^{a}P < 0.05$; NS: Not significant; eNOS: Endothelial nitric oxide synthase; PVL: Portal vein ligation; ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy.

only in patients who underwent ALPPS, and this evaluation was not compared with that in patients who underwent PVE. Furthermore, the transition of HA flow before and after ALPPS has not been evaluated in a rat model. In the rat ALPPS model, liver transection between the right and left median lobes with ligation of the Glisson of the left median lobe caused a necrotic change in the left median lobe, which is synonymous with liver resection of the left median lobe considering hemodynamics. These results suggest that both hemodynamic changes and differences in oxygenation of the FLR affect regeneration rates in the ALPPS and PVL models. The increased HA flow to the RML observed in the



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Figure 6 Changes in liver regeneration and cell proliferation due to drug administration. A: Future liver remnant/body weight (FLR/BW) ratio in the N-nitro-arginine methyl ester (L-NAME)-administered associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) group; B: Ki-67 labeling index in the L-NAME-administered ALPPS group; C: FLR/BW ratio in the molsidomine-administered portal vein ligation (PVL) group; D: Ki-67 labeling index in the molsidomine-administered PVL group; n = 5 for each group; $^{a}P < 0.05$; NS: Not significant; PVL: Portal vein ligation; ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy; FLR/BW: Future liver remnant/body weight; L-NAME: N-nitro-arginine methyl ester.



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Figure 7 Evaluation of hepatic artery and portal vein volumetric blood flow in the future liver remnant after surgery. A: Portal vein (PV) flow in control, PV ligation (PVL), and associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) groups; B: Hepatic artery flow in control, PVL, and ALPPS groups; C: Total blood flow in control, PVL, and ALPPS groups. Values are expressed as the mean ± SD; n = 4 for each group; $^{a}P < 0.05$; NS: Not significant; PVL: Portal vein ligation; ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy; HA: Hepatic artery.

ALPPS group may have been due to a hepatic arterial buffer response derived from collateral blood flow blockage by hepatectomy. In contrast, the reason for the observed decrease in HA flow to the RML in the PVL group might be the effect of HA influx from the RML to the left median lobe (LML) *via* collateral circulation after the PV blockade to the LML.

This study had some limitations. First, because we observed short-term changes in rat models, it is unknown whether NO activation promotes clinically meaningful liver regeneration in humans. Second, the mechanism underlying the activation of the Akt-eNOS pathway is unclear and requires further investigation that includes real-time monitoring of oxygenation in the FLR. Despite these shortcomings,

we believe that our results are of interest because few reports have focused on the relationship between eNOS activation and liver regeneration after ALPPS.

CONCLUSION

The activation of the Akt-eNOS pathway in ALPPS may be an important factor in promoting early liver regeneration. If a combination of NO-producing agents and PVL or PVE enables liver regeneration within a short time after surgery, it may be an alternative to ALPPS and is expected to be applied clinically as a less invasive procedure.

ARTICLE HIGHLIGHTS

Research background

Associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) has already been clinically applied in various countries. Although it has been reported that ALPPS offers faster and larger liver regeneration compared to portal vein embolization (PVE), the mechanism of this phenomenon is still unclear.

Research motivation

The aim of this study was to investigate the underlying mechanism of rapid liver regeneration after ALPPS focusing on inflammatory cytokines and endothelial nitric oxide synthase (eNOS) activation.

Research objectives

Activation of eNOS was considered one of key points on mechanism of rapid liver regeneration after ALPPS.

Research methods

Liver regeneration was compared between the rat portal vein ligation (PVL) model and the rat ALPPS model. In addition, impact of administration of gadolinium chloride ($GdCl_3$, Kupffer cell inhibitor), NG-nitro-arginine methyl ester (L-NAME, NOS inhibitor), and molsidomine (NO enhancer) on liver regeneration after PVL and/or ALPPS.

Research results

Administration of $GdCl_3$ before ALPPS provided no significant negative influence of liver regeneration after ALPPS. Administration of L-NAME before ALPPS suppressed liver regeneration after ALPPS, while administration of molsidomine before PVL accerelated liver regeneration after PVL as well as ALPPS.

Research conclusions

ALPPS is an alternative to PVE for reducing posthepatectomy liver failure after major hepatectomy.

Research perspectives

Combination of NO-producing agents and less invasive procedure can be an alternative to ALPPS procedure in the future.

FOOTNOTES

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Country/Territory of origin: Japan

ORCID number: Hitoshi Masuo 0000-0003-2930-7156; Akira Shimizu 0000-0002-5015-1697; Hiroaki Motoyama 0000-0003-0882-2626; Koji Kubota 0000-0003-0164-7685; Tsuyoshi Notake 0000-0002-2277-5347; Takahiro Yoshizawa 0000-0001-7624-9257; Kiyotaka Hosoda 0000-0001-6928-3676; Koya Yasukawa 0000-0002-3643-0921; Akira Kobayashi 0000-0002-9161-0159; Yuji Soejima 0000-0001-5539-9280.

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ORIGINAL ARTICLE

Retrospective Study

Convolutional neural network-based segmentation network applied to image recognition of angiodysplasias lesion under capsule endoscopy

Ye Chu, Fang Huang, Min Gao, Duo-Wu Zou, Jie Zhong, Wei Wu, Qi Wang, Xiao-Nan Shen, Ting-Ting Gong, Yuan-Yi Li, Li-Fu Wang

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Ye Chu, Duo-Wu Zou, Jie Zhong, Wei Wu, Qi Wang, Xiao-Nan Shen, Ting-Ting Gong, Li-Fu Wang, Department of Gastroenterology, Shanghai Jiao Tong University School of Medicine, Ruijin Hospital, Shanghai 200025, China

Fang Huang, Min Gao, Yuan-Yi Li, Technology Platform Department, Jinshan Science & Technology (Group) Co., Ltd., Chongqing 401120, China

Corresponding author: Li-Fu Wang, MD, PhD, Chief Physician, Professor, Department of Gastroenterology, Shanghai Jiao Tong University School of Medicine, Ruijin Hospital, No. 197 Ruijin Er Road, Shanghai 200025, China. lifuwang@sjtu.edu.cn

Abstract

BACKGROUND

Small intestinal vascular malformations (angiodysplasias) are common causes of small intestinal bleeding. While capsule endoscopy has become the primary diagnostic method for angiodysplasia, manual reading of the entire gastrointestinal tract is time-consuming and requires a heavy workload, which affects the accuracy of diagnosis.

AIM

To evaluate whether artificial intelligence can assist the diagnosis and increase the detection rate of angiodysplasias in the small intestine, achieve automatic disease detection, and shorten the capsule endoscopy (CE) reading time.

METHODS

A convolutional neural network semantic segmentation model with a feature fusion method, which automatically recognizes the category of vascular dysplasia under CE and draws the lesion contour, thus improving the efficiency and accuracy of identifying small intestinal vascular malformation lesions, was proposed. Resnet-50 was used as the skeleton network to design the fusion mechanism, fuse the shallow and depth features, and classify the images at the pixel level to achieve the segmentation and recognition of vascular dysplasia. The training set and test set were constructed and compared with PSPNet, Deeplab3+, and UperNet.

RESULTS

The test set constructed in the study achieved satisfactory results, where pixel accuracy was 99%, mean intersection over union was 0.69, negative predictive value was 98.74%, and positive predictive value was 94.27%. The model parameter was 46.38 M, the float calculation was 467.2 G, and the time length to segment and recognize a picture was 0.6 s.

CONCLUSION

Constructing a segmentation network based on deep learning to segment and recognize angiodysplasias lesions is an effective and feasible method for diagnosing angiodysplasias lesions.

Key Words: Artificial intelligence; Image segmentation; Capsule endoscopy; Angiodysplasias

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Core Tip: Small intestinal vascular malformation (vascular dysplasia) is a common cause of small intestinal bleeding. Herein, we proposed a semantic recognition segmentation network to recognize small intestinal vascular malformation lesions. This method can assist doctors in identifying lesions, improving the detection rate of intestinal vascular dysplasia, realizing automatic disease detection, and shortening the capsule endoscopy reading time.

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INTRODUCTION

Small intestinal vascular malformations (angiodysplasias) are common causes of small intestinal bleeding[1,2]. Angiodysplasias are degenerative lesions that manifest as abnormalities of arteries, veins, or capillaries of the original normal blood vessels. Occasionally, the term angiodysplasias include various synonymous disease concepts, such as angioectasia (AE), Dieulafoy's lesion (DL), and arteriovenous malformation. According to the Yano-Yamamoto classification, small bowel vascular lesions are classified into four types under endoscopy[3]. AE includes small erythemas and can be defined as type 1a: punctuate (< 1 mm), or type 1b: patchy (a few mm). They are characterized by thin, dilated, and tortuous veins lacking smooth muscle layers, which explain their weakness and tendency to bleed. Typically, DLs consist of small mucosal defects and can be classified as type 2a: punctuate lesions with pulsatile bleeding or type 2b: pulsatile red protrusions without surrounding venous dilatation[4]. Some arteriovenous malformations and pulsatile red protrusions with dilated peripheral veins are defined as type 3. Congenital intestinal arteriovenous malformations manifest as polypoid or cluster type[5,6] and are classified as type 4. Nevertheless, the Yano-Yamamoto classification cannot fully reflect the histopathological findings.

Capsule endoscopy (CE) is a painless and well-tolerated approach that can achieve complete visualization of the small intestine[7]. It captures images for > 8 h[8]. Previous studies have demonstrated the probability of CE diagnosis of angiodysplasias was 30%-70%, and > 50% of obscure gastrointestinal bleeding patients have angiodysplasias[9-11]. The detection rate of CE was reported to be higher than other diagnostic methods, such as small bowel computed tomography, mesenteric angiography, and enteroscopy. Therefore, using CE as a first-line inspection tool for the diagnosis of angiodysplasias is recommended[12]. Nonetheless, CE has some limitations, and only 69% of angiodysplasias can be diagnosed by gastroenterologists[13]. Less relevant lesions, such as erosions or tiny red spots, are regarded as negative results; however, distinguishing highly relevant lesions from less relevant lesions could be challenging. In addition, the diagnostic efficiency of CE decreases when the presence of bile pigments, food residues, or bubbles affects the observation of the intestinal mucosa. The doctor's manual reading of the entire gastrointestinal tract is time-consuming, and the heavy workload affects the accuracy of the diagnosis. Therefore, making diagnosis of angiodysplasias solely based on CE is challenging.

The detection rate of angiodysplasias in the small intestine can be increased by using artificial intelligence (AI) to assess the effect of automatic diagnosis, which has been successfully applied for the recognition and diagnosis of gastrointestinal endoscopic images[14]. AI assists in the recognition and diagnosis of CE images, eliminates errors in manual reading, reduces the workload of doctors, and

improves diagnosis efficiency. The clinical application of AI-based deep learning technology in wireless CE has been a research focus, which has gained increasing interest in the past two years[15-32]. Several studies[15,23-26] have used deep learning to identify ulcers from CE data. Pogorelov *et al*[27] used the color texture features to detect small intestinal bleeding in CE data. Blanes-Vidal *et al*[28] constructed a classification network to identify intestinal polyp lesions in CE data. Kundu *et al*[29] and Hajabdollahi *et al*[30] identified small bowel bleeding in CE data using a classification neural network. Obscure gastrointestinal bleeding is the main indication for small intestinal CE, and the potential risk of bleeding from vascular malformations is high[14]. Therefore, we focused on AI-assisted recognition technology for angiodysplasias in the present study. Hitherto, there are few semantic segmentation networks based on deep learning to segment and recognize angiodysplasias lesions in CE, which prompted us to introduce a segmentation model in the study. Compared with the classification model and target detection model in deep learning, the segmentation model based on deep learning can more accurately locate the focus of small intestinal vascular malformation, better assist doctors in diagnosing small intestinal vascular malformation, and improve the accuracy and efficiency of doctors' diagnosis.

Currently, significant progress has been made in semantic segmentation in the field of deep learning. To the best of our knowledge, this is the first paper that proposed using a semantic segmentation network to solve the pixel-level small intestinal vascular malformation focus recognition and location. Resnet-50 was used as the skeleton network, and the fusion mechanism based on shallow features and deep features was introduced so that the segmentation model could accurately locate the location and category of lesions. Shallow features can perceive the texture details of lesions, while deep features can perceive the semantic information between lesions. By combining these two features to segment the image, the phenomenon where the lesion area is divided into uncorrelated small areas is reduced, the pixel accuracy (PA) is improved, and the missed detection rate of the lesion is reduced. This paper introduced the proposed network structure in detail and compared three common segmentation models, *i.e.*, PSPNet[31], Deeplabv3+[32], and UperNet[33]. The obtained results confirmed that the model proposed in this paper had high-performance indicators.

MATERIALS AND METHODS

ResNet was introduced in 2015 and won first place in the classification task of the ImageNet competition on account of being "simple and practical". Afterward, many methods, which were based on ResNet50 or ResNet101, have been widely used in detection, segmentation, recognition, and other fields. This method makes a reference (X) for the input of each layer, learning to form residual functions rather than learning some functions without reference (X). This residual function is easier to optimize and can greatly deepen the number of network layers. Moreover, the extracted image features have strong robustness. ResNet50 is faster than ResNet100. Therefore, ResNet50 is selected as the skeleton network of the semantic segmentation network in this paper. Based on the fusion of shallow and deep features, Resnet-50 was used as the skeleton network to construct an improved convolutional neural network (CNN) segmentation network model that automatically recognizes the type of angiodysplasias under CE and draws the outline of the lesion in the study. The present study aimed to assist doctors in diagnosing angiodysplasias lesions with CE.

The model proposed in this study was composed of three sub-units, *i.e.*, down-sampling, up-sampling, and classifier. CE small intestine data were used as input in the module, and the final output was image lesion category information and lesion boundary information.

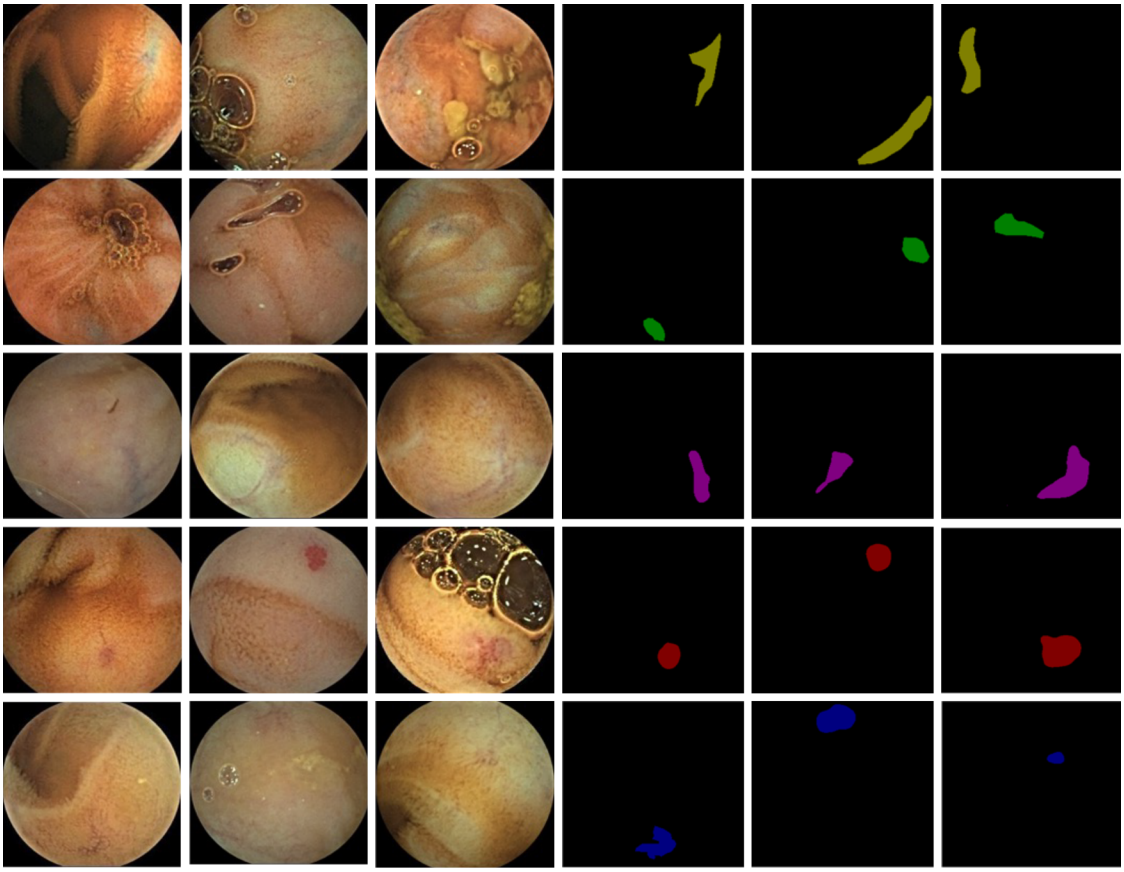
Research data set

In order to train and evaluate the segmentation model, 378 patients with angiodysplasias who underwent OMOM CE (China Chongqing Kingsoft Technology Co., Ltd) at the Ruijin Hospital between January 2014 and December 2020 were recruited in this study. The sampling frequency of OMOM capsules of 2fps, the working time of > 12 h, and the apex field of view of 150° were used to diagnose the patients. A total of 12403 pictures were identified with an image resolution of 256 × 240. The patient data were anonymized, any personal identification information was omitted, and examination information (such as examination date and patient name) was deleted from the original image. All patients provided written informed consent, and the ethics committee approved the study [the certification number was (2017) provisional ethics review No. 138]. The annotated data were marked by an experienced endoscopy group that included three experts from Ruijin Hospital Affiliated to Shanghai Jiao Tong University. The average age of the experts was 35 years, and their average CE reading experience was 5 years, with an average of 150 CE cases each year. The five types of lesions of vascular malformation were annotated, and 12403 image data and 12403 annotated mask image data were generated. The data sample map is shown in Figure 1.

This project used the image data of 178 cases as the training set and the remaining 200 cases as the test set. The training set was divided into training and verification data at a ratio of 7:3 during the training process. The test set contained 1500 images without lesions and 1500 images with lesions. The training set and test set image data are summarized in Table 1.

Table 1 Details of the training set data and test set data

Lesion type	Lesion morphology	Number of pictures/pieces	
		Training set	Test set
Telangiectasia	Red cluster	838	38
	Red spider nevus	162	4
Venous dilatation	Red branched	752	38
	Blue branched	2583	1088
Vein tumor	Blue cluster	3058	332



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Figure 1 Sample image of training data. The left three columns are the original image of the capsule, and the right three columns are the manual annotation results.

Data preprocessing

The training data were preprocessed to meet the requirements of the deep learning model. The preprocessing steps of the model constructed in the study were as follows: (1) Resizing the image to 256 × 240 × 3; (2) using enhancement methods (rotation, flip, and tilt) on the resized image; and (3) normalizing all images. In order to train a deep learning model, the dataset was split. The dataset image was randomly divided into two parts: 70% for training and 30% for verification.

Segmentation network details

The network structure proposed in this study is shown in Figure 2. The construction of the network model was inspired by the UperNet model. ResNet-50 was used as the skeleton network. The fusion mechanism of shallow features and deep features were introduced. Subsequently, the feature with the same size as the original image was obtained through the down-sampling operation. Finally, the classifier was connected to realize the pixel-level segmentation task of the image.

Based on the new semantic segmentation recognition network framework, a single end-to-end network could be trained to capture and analyze the semantic information of the CE small intestine

data. In order to fuse the shallow features and deep feature information, the last feature mapping set output by each stage in ResNet was expressed as C1, C2, C3, and C4, and the two-by-two fusion of features were utilized as down-sampling operation input, where the down-sampling rates were 4, 8, 16, and 32, respectively. The texture features of the lesion were captured at the highest layer, and the pixel-level segmentation of the lesion was completed based on the lowest layer features.

The last down-sampling operation generated a feature map with the same resolution as the original image, with a size of 256 × 240. After the feature was operated by Flatten, a classifier composed of a fully connected layer was connected to complete the segmentation and recognition tasks of the capsule data.

In order to assess the fusion of features of different scales, bilinear interpolation was used to adjust them according to the size, after which a non-evolutionary layer was applied to fuse the features of different levels and reduce the channel size. All non-classifier convolutional layers underwent batch normalization Relu operations after output. The learning rate of the current iteration was equivalent to the initial learning rate multiplied by (1-iter/max-iter_size)^{power}, and the initial learning rate and power were set to 0.02 and 0.9, respectively.

RESULTS

Evaluation index

The performance of the segmentation model of angiodysplasias lesions in CE was evaluated based on the following indicators: Positive predictive value (PPV), negative predictive value (NPV), mean intersection over union (mIOU), and PA. PPV and NPV were calculated using formulae 1 and 2, respectively.

$$PPV = \frac{TP}{TP + FP} \quad (1)$$

$$NPV = \frac{TN}{TN + FN} \quad (2)$$

Where true positive (TP) and true negative (TN) are the true number of positive samples and the true number of negative samples, respectively; FP and FN are false positives and false negatives, respectively. IOU and mIOU calculation formulae are shown as formulae 3 and 4, respectively.

$$OU = \frac{\text{Target area} \cap \text{Prediction area}}{\text{Target area} \cup \text{Prediction area}} \quad (3)$$

$$mIOU = \frac{1}{k+1} \sum_{i=0}^{k} \frac{P_{ii}}{\sum_{j=0}^{k} P_{ij} + \sum_{j=0}^{k} P_{ji} - P_{ii}} \quad (4)$$

Supposedly, there were K+1 categories (including an empty category or background) in semantic segmentation, which indicated that class i is predicted as i, and class j is predicted as j. The PA is calculated by formula 5.

$$PA = \frac{\sum_{i=0}^{k} P_{ii}}{\sum_{i=0}^{k} \sum_{j=0}^{k} P_{ij}} \quad (5)$$

Experimental design

Python 3 is a good deep-learning programming language that supports multiple deep-learning frameworks. The model was implemented using Python 3 and Torch framework. The training server has a graphics processing unit. All images were first passed to the image data generation class in Pytorch, and the preprocessing operations were performed, including enhancement, resize, and normalization operations. Then, the generated images were sent to the model to start the training. The layers in the backbone network ResNet-50 used pre-trained weights on ImageNet. An optimizer (SGD) was used to train the model, after which a weight decay of 0.0001 and a momentum of 0.9 were applied. Each model ran approximately 25000 Epochs; each Epoch iterated eight times, and the batch size was 8. In the model training process, the loss change, PA index, and mIOU changes were detected (Figure 3).

Comparison results of multiple models

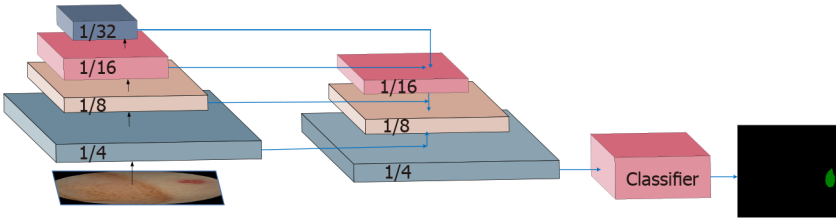
On a test set consisting of 3000 image data, the following test indicators were compared on the four models: PPV, NPV, mean IOU, PA, parameter quantity, float calculation quantity, and duration. The results are shown in Table 2.

Based on the method of fusion of shallow and deep features, the CNN segmentation network model was improved and optimized, and the segmentation and recognition of five types of angiodysplasias lesions, *i.e.*, blue branch, blue cluster, red branch, red cluster, and red spider nevus, were realized. This method fully uses the shallow and deep features extracted from the skeleton network to perceive the global information and lesion texture information of the small intestine capsule image data as a whole. Thus, it significantly improves the PPV and NPV of the segmentation model in the angiodysplasias lesion image. In order to obtain the highest PPV, the NPV has to be the highest. The unified perception of the global and local information of the small intestine capsule data was completed through a CNN,

Table 2 Comparison of model accuracy

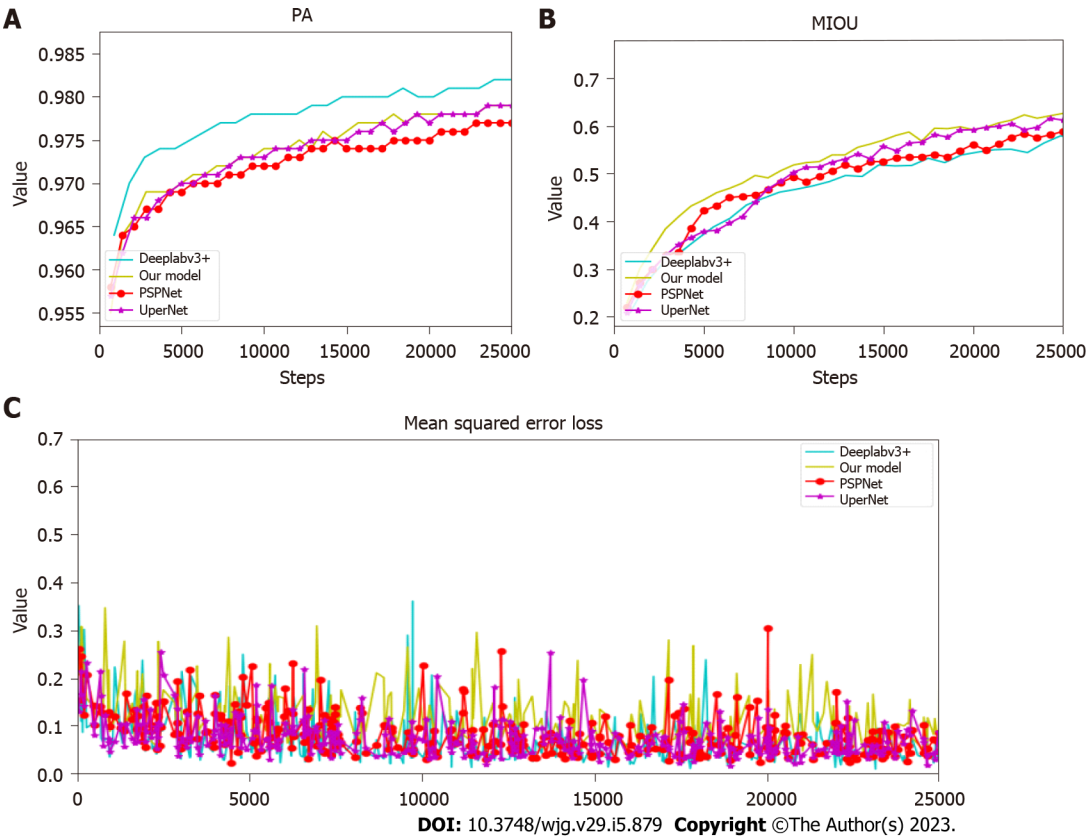
Network type	PPV (%)	NPV (%)	mIOU	PA (%)	Parameter (M)	Float calculation amount (G)	Time (s)
PSPNet	85.14	98.62	0.64	98	51.43	829.10	0.9
DeeplabV3+	45.07	99.75	0.59	89	59.34	397.00	0.95
UperNet	92.55	95.69	0.69	98	126.08	34.94	0.9
Our model	94.27	98.74	0.69	99	46.38	467.2	0.6

PPV: Positive predictive value; NPV: Negative predictive value; mIOU: Mean intersection over union; PA: Pixel accuracy.



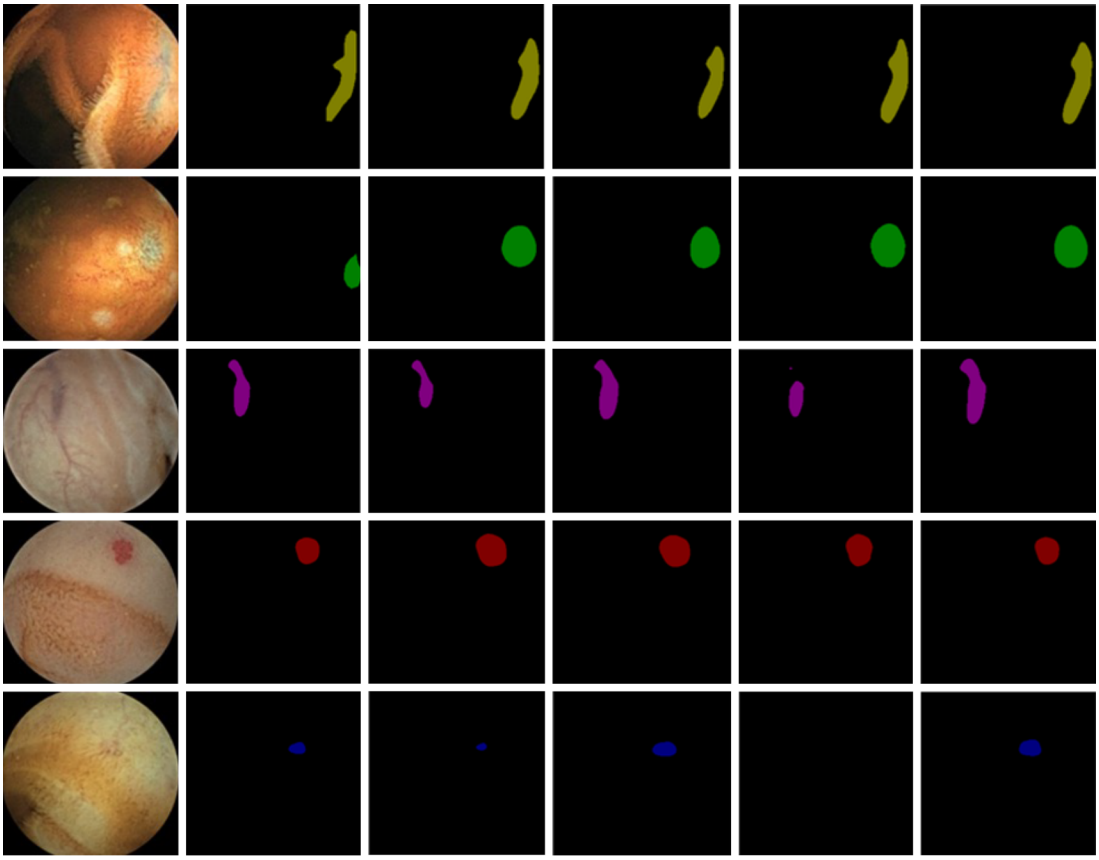
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Figure 2 Network structure diagram. This figure shows the structure of semantic segmentation network, in which the first two modules are shallow and deep feature fusion, the third module is pixel classifier, and finally the network output results.



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Figure 3 Change process of loss, pixel accuracy and mean intersection over union. A: Comparation of the pixel accuracy (PA) values of each model during training. The abscissa represents the number of training iterations, and the ordinate represents the value of PA; B: Comparation of the mean intersection over union (mIOU) values of each model during training. The abscissa represents the number of training iterations, and the ordinate represents the value of mIOU; C: Comparation of the loss value of each model in the training process. The abscissa represents the number of training iterations, and the ordinate represents the loss value. PA: Pixel accuracy; mIOU: Mean intersection over union.



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Figure 4 Five rows from top to bottom: blue branch, blue lumpy, red branch, red lumpy and red spider nevus. The first column on the left is the original image, the middle four columns are the results of the current excellent segmentation network, and the last column is the results of the model we proposed.

which reduced the number of network model parameters, the number of float calculations, and the inference time of the deep learning model. Furthermore, a comparative experiment was designed and compared to the current advanced segmentation network models: PSPNet, DeeplabV3+, and UperNet. Our model showed that the NPV reached the highest 98.74% when the PPV was the highest.

The comparison of the segmentation and recognition effects of the four models on the vascular aberration lesions of the CE small intestine data is shown in Figure 4. The model proposed in the study was similar to that of the expert's annotation results.

Compared with relevant literature, Leenhardt *et al*[19] applied technology for segmentation, achieving the highest level of lesion detection, with an NPV value of 96%. However, the algorithm presented in this paper had some advantages in the test set. Also, our NPV value was 98%.

DISCUSSION

The classification network and the target detection network are the mainstream network structure that combines the deep learning model and the CE diagnosis method. In the present study, we introduced the segmentation network in deep learning, segmented and identified the angiodysplasias lesions, and completed the pixel-level segmentation task of the angiodysplasias lesions. The semantic segmentation network model had clinical practicality application as assessed using the training and test sets in comparative experiments.

The segmentation networks have been obviously developed in the field of deep learning. PSPNet uses the prior knowledge of the global feature layer to understand the semantics of various scenes, combined with the deep supervision loss to develop an effective optimization strategy on ResNet and embed difficult-to-analyze scene information features into the functional connectivity networks prediction framework to establish a pyramid. The pooling module aggregates the contextual information in different regions and improves the ability to obtain global information. This system was used for scene analysis and semantic segmentation and was 83% accurate on the COCO data set. The DeepLabV3+ model was based on an encoder-decoder structure, which improved the accuracy and saved the inference time; an accuracy rate of 89% was obtained in the COCO dataset. UperNet used

unified perception analysis to build a network with a hierarchical structure to ensure that multiple levels were resolved at visual concepts, learn the differentiated data in various image datasets, achieve joint reasoning, and explore the rich visual knowledge in the images. Finally, 79.98%-PA was obtained on the ADE20K data set. UperNet used a unified perception analysis module from scenes, objects, parts, materials, and textures to simultaneously analyze the multilevel visual concepts of images, such that many objects could be segmented and recognized, and the rate of missed objects could be reduced. The CE small intestine image data has a simple scene and fewer semantic levels. The use of large segmentation network models would cause over-fitting in training and high computational complexity. This study was inspired by UperNet and optimized basic CNN segmentation network, which led to the creation of a network model suitable for the segmentation and recognition of angiodysplasias with CE.

On the other hand, a case-based dataset encompassing typical vascular malformation images, atypical angiodysplasias images, and normal images was constructed, including pictures with poor intestinal cleanliness. According to the color and morphology of the angiodysplasias lesions in the cases, the five types of angiodysplasias lesions were summarized as blue branched, blue cluster, red branched, red cluster, and red spider nevus. The dataset constructed in this study verified the clinical applicability of the semantic segmentation model. Thus, the dataset was essential in diagnosing CE small bowel vascular malformation based on the deep learning model.

CONCLUSION

The deep learning model constructed in this study showed high PPV and NPV for the segmentation and recognition of angiodysplasias lesions. In the future, it could be used to assist capsule endoscopists in the real-time diagnosis of angiodysplasias lesions. Deep learning does not require prior knowledge, as it can directly learn the most predictive features from image data, as well as segment and recognize the image. The larger the amount of data, the higher the advantages of deep learning and the higher the recognition accuracy. AI facilitates grassroots' CE to obtain the same diagnosis effect as senior experts. However, the current uneven distribution of medical resources and the technical level of grassroots CE are the driving forces for the development of AI. In conclusion, the segmentation model based on deep learning can assist doctors in identifying the lesions of small intestinal vascular malformations.

ARTICLE HIGHLIGHTS

Research background

Small intestinal vascular malformations (angiodysplasias) commonly cause small intestinal bleeding. Therefore, capsule endoscopy has become the primary diagnostic method for angiodysplasias. Nevertheless, manual reading of the entire gastrointestinal tract is a time-consuming heavy workload, which affects the accuracy of diagnosis.

Research motivation

The doctor's manual reading of the entire gastrointestinal tract is time-consuming, and the heavy workload affects the accuracy of the diagnosis. Also, significant progress has been made in semantic segmentation in the field of deep learning.

Research objectives

This study aimed to assist in the diagnosis and increase the detection rate of angiodysplasias in the small intestine, achieve automatic disease detection, and shorten the capsule endoscopy (CE) reading time.

Research methods

A convolutional neural network semantic segmentation model with feature fusion automatically recognizes the category of vascular dysplasia under CE and draws the lesion contour, thus improving the efficiency and accuracy of identifying small intestinal vascular malformation lesions, was proposed.

Research results

The test set constructed in the study achieved satisfactory results: pixel accuracy was 99%, mean intersection over union was 0.69, negative predictive value was 98.74%, and positive predictive value was 94.27%. The model parameter was 46.38 M, the float calculation was 467.2 G, and the time needed to segment and recognize a picture was 0.6 s.

Research conclusions

Constructing a segmentation network based on deep learning to segment and recognize angiodysplasias

lesions is an effective and feasible method for diagnosing angiodysplasias lesions.

Research perspectives

The model detects the small intestinal malformation lesions in the capsule endoscopy image data and draws the lesion area through segmentation.

FOOTNOTES

Author contributions: Chu Y read and reviewed capsule endoscopy imagines, and participated in the design, writing, and revision of the manuscript; Huang F performed AI algorithm research, designed the segmentation recognition protocol, and verified the algorithm; Gao M organized the experimental results, visualized the experiment, and wrote the draft of the paper; Zou DW designed and revised the protocol of the paper; Zhong J reviewed capsule endoscopy images; Wu W and Wang Q participated in the reading of CE and preparation of the material; Shen XN and Gong TT partial writing of the manuscript; Li YY contributed to the coding and debugging of interface between algorithm and software; Wang LF designed and modified the protocol of the paper.

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Country/Territory of origin: China

ORCID number: Ye Chu 0000-0002-9204-9498; Fang Huang 0000-0002-8758-8534; Min Gao 0000-0002-4969-2488; Duo-Wu Zou 0000-0002-2461-5304; Jie Zhong 0000-0003-2919-1771; Wei Wu 0000 0001 7842 9032; Qi Wang 0000-0002-8437-8224; Xiao-Nan Shen 0000-0002-4169-3355; Ting-Ting Gong 0000-0003-1298-787X; Yuan-Yi Li 0000-0002-3180-7398; Li-Fu Wang 0000-0001-5172-9932.

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ORIGINAL ARTICLE

Clinical Trials Study

Efficacy of dexamethasone and N-acetylcysteine combination in preventing post-embolization syndrome after transarterial chemoembolization in hepatocellular carcinoma

Nitipon Simasingha, Wasu Tanasoontrarat, Torpong Claimon, Supatsri Sethasine

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Nitipon Simasingha, Supatsri Sethasine, Department of Internal Medicine, Navamindradhiraj University, Bangkok 10300, Thailand

Wasu Tanasoontrarat, Torpong Claimon, Department of Radiology, Navamindradhiraj University, Bangkok 10300, Thailand

Corresponding author: Supatsri Sethasine, MD, Associate Professor, Chief Doctor, Department of Internal Medicine, Navamindradhiraj University, Vajira Hospital Dusit Thailand, Bangkok 10300, Thailand. supatsri@nmu.ac.th

Abstract

BACKGROUND

Conventional transarterial chemoembolization (cTACE) is the current standard treatment for intermediate-stage hepatocellular carcinoma (HCC). Post-embolization syndrome (PES) is complex clinical syndrome that presents as fever, abdominal pain, nausea, and vomiting. Either dexamethasone (DEXA) or N-acetylcysteine (NAC) is used to prevent PES; however, the synergistic effect of their combined therapy for preventing PES and liver decompensation has not been determined.

AIM

To evaluate the efficacy of DEXA and NAC combination in preventing PES and liver decompensation after cTACE.

METHODS

Patients with Barcelona Clinic Liver Cancer stage A or B HCC who were scheduled for TACE were prospectively enrolled. All patients were randomly stratified to receive NAC and DEXA or placebo. The dual therapy (NAC + DEXA) group received intravenous administration of 10 mg DEXA every 12 h, NAC 24 h prior to cTACE (150 mg/kg/h for 1 h followed by 12.5 mg/kg/h for 4 h), and a continuous infusion of 6.25 mg/h NAC plus 4 mg DEXA every 12 h for 48 h after cTACE. The placebo group received an infusion of 5% glucose solution until 48 h after procedure. PES was defined by South West Oncology Group toxicity code grading of more than 2 that was calculated using incidence of fever, nausea, vomiting, and pain.

RESULTS
One-hundred patients were enrolled with 50 patients in each group. Incidence of PES was significantly lower in the NAC + DEXA group compared with in the placebo group (6% *vs* 80%; $P < 0.001$). Multivariate analysis showed that the dual treatment is a protective strategic therapy against PES development [odds ratio (OR) = 0.04; 95% confidence interval (CI): 0.01-0.20; $P < 0.001$). Seven (14%) patients in the placebo group, but none in the NAC + DEXA group, developed post-TACE liver decompensation. A dynamic change in Albumin-Bilirubin score of more than 0.5 point was found to be a risk factor for post-TACE liver decompensation (OR = 42.77; 95%CI: 1.01-1810; $P = 0.049$).

CONCLUSION
Intravenous NAC + DEXA administration ameliorated the occurrence of PES event after cTACE in patients with intermediate-stage HCC.

Key Words: Hepatocellular carcinoma; Post-embolization syndrome; Transarterial chemoembolization; Liver decompensation

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Core Tip: Conventional transarterial chemoembolization (TACE) is the current standard treatment for intermediate-stage hepatocellular carcinoma (HCC). A combination of N-acetylcysteine and dexamethasone ameliorated the occurrence of post-embolization syndrome event after TACE in patients with intermediate-stage HCC. A dynamic change in Albumin-Bilirubin score of more than 0.5 point was found to be a risk factor for post-TACE liver decompensation.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a major-public health concern and the fourth common cause of cancer-related deaths worldwide[1]. In Thailand, HCC is the second most common tumor type and the most common cause of cancer-related death. Without treatment, patients with HCC have a one-year overall survival rate of less than 20%. Conventional transarterial chemoembolization (cTACE) has been established as a standard treatment for HCC with Barcelona Clinic Liver Cancer (BCLC) stage B. Systemic reviews and meta-analyses have demonstrated that cTACE therapy improved the survival of patient at this stage. HCC patients who underwent super-selective TACE had a 5-year survival rate of 40%-48%. cTACE involves embolization of vessel supply to tumors, causing ischemia in not only tumor cells but also normal hepatocytes, along with targeted chemotherapy; the systemic effects of chemotherapeutic agents result in the occurrence of post-embolization syndrome (PES)[2-5].

PES is a complex clinical syndrome manifested as fever, abdominal pain, nausea, and vomiting[6,7] with a South West Oncology Group (SWOG) score > 2[7]. PES is self-limited and either resolves within 24 h or exhibits sustained symptoms for up to two weeks based on various factors such as tumor size, tumor numbers, dosage of chemotherapy, and performance status of the patient[6,8,9]. Management of PES mainly includes supportive treatment such as with analgesic, antiemetic, and antipyretic administration[9,10]. Depending on its pathogenesis, PES may be related to systemic inflammation, resulting in toxic and ischemic effects on tumor cells and hepatocytes[9]. Steroids and antioxidants may play an important role in the prevention and treatment of PES. Dexamethasone (DEXA) and prednisolone are recommended by the National Comprehensive Cancer Network as effective medications for preventing chemotherapy-induced nausea/vomiting. Moreover, a few randomized control trials have demonstrated the effect of DEXA in terms of PES prevention[11-14]. N-acetylcysteine (NAC), an established glutathione precursor and a potent antioxidant, was found to ameliorate ischemic liver injury by improving the systemic hemodynamic parameter and tissue oxygen delivery in animal models[14-16]. Further, a pilot study reported that NAC can reduce the incidence of PES after cTACE but cannot reduce that of post-TACE liver decompensation[17]. Currently, there are no standard prevention guidelines for PES. Our study aims to evaluate the dual effect of DEXA and NAC therapy in preventing

PES or liver decompensation after cTACE in patients with HCC.

MATERIALS AND METHODS

Study design

A randomized, double blind, placebo-controlled trial was conducted at the Gastroenterology and Liver Unit, Vajira Hospital, Navamindradhiraj University, Bangkok, Thailand from November 2020 to January 2022.

Study population

Eligible patients were those aged 18-80 years with diagnosed early- or intermediate-stage HCC, according to BCLC classification, and had a good performance status, defined by the Eastern Cooperative Oncology Group. Diagnosis of HCC was based on either histological or radiological typical hallmark criteria according to the American Association for the Study of Liver Disease[18] and European Association for the Study of the Liver[19]. The exclusion criteria were as follows: (1) Decompensated liver cirrhosis (Child-Pugh score ≥ 9) or cirrhosis with main portal vein invasion; (2) Congestive heart failure and/or respiratory failure; (3) Severe comorbid illness, such as end-stage renal disease, persistent poorly-controlled diabetes mellitus or hemoglobin A1C ≥ 8.5, uncontrolled hypertension (systolic blood pressure ≥ 180 mmHg or diastolic blood pressure ≥ 120 mmHg), with a life expectancy of < 6 mo; (4) Severe allergy or anaphylaxis/anaphylactoid to NAC; (5) Pregnancy; and (6) History of non-steroid anti-inflammatory drugs, steroids, or NAC use within 21 d of trial initiation. All enrolled patients agreed on receiving cTACE treatment and provided informed consent before participating in the study.

Sample size calculation

Based on previous results, the incidence of PES among patients with HCC after receiving cTACE was > 60%[12]. Superiority of the DEXA regimen over the control regimen was defined as a 25% decrease in PES. Intravenous DEXA was hypothesized to reduce the incidence of PES by 20%. This study used a two-tailed test that calculated the requirement of at least 44 patients in each group to obtain a P value < 0.05 with alpha and beta errors of 5% and 20%, respectively.

Ethical approval

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Board of Faculty of Medicine, Vajira Hospital (COA 051/2564).

Randomized strategy and intervention

All patients were admitted at least 24 h before the prescheduled cTACE procedure and were randomly (1:1) assigned to either NAC–DEXA or placebo group. The randomization sequence was computer-generated in blocks of four and stratified according to Child-Pugh classification (class A or B). All patients were blinded to the treatment assignment. Both groups underwent therapy initiation 24 h prior to the procedure. The specific dosage of the NAC-DEXA protocol was based on a previously reported recommended dosage[11,17]. The NAC-DEXA group received intravenous infusion of 5% dextrose with NAC, with an initial loading dose of 150 mg/kg/h over 1 h followed by 12.5 mg/kg/h for 4 h and 10 mg of intravenous DEXA every 12 h; this was followed by continuous intravenous infusion of 6.25 mg/kg/h NAC and 4 mg of intravenous DEXA every 12 h for the remaining 48 h post-TACE. The placebo group received 5% glucose in normal saline for 48 h at an infusion rate of 60 mL/h post-TACE (as shown in Supplementary Figure 1). If mild-to-moderate allergic symptoms developed (*e.g.*, urticarial rash or bronchospasm), treatment was temporarily stopped for 1 h and intravenous antihistamine was immediately administered; treatment was resumed after the symptoms subsided. If severe allergic or anaphylactoid reaction occurred, treatment was permanently stopped, and the patient was treated according to standard protocol for severe allergic reaction. cTACE was performed by two interventional radiologists (Tanasoontrarat W and Claimon T) who were blinded to the randomization assignment. Pre-procedure single intravenous dose of ceftriaxone (1 g) or amoxicillin-clavulanic acid (1.2 g) along with single intravenous dose of ondansetron (8 mg) was administered to all patients. The femoral artery was catheterized under local anesthesia. A thorough angiographic examination was performed to locate all of the tumor-feeding arteries. An emulsion of lipiodol (2.5-15 mL) and chemotherapeutic agent (mitomycin, 5-20 mg) was infused into the feeders at an optimal dose determined by the interventional radiologist to be sufficient for tumor control. Thereafter, gelatin sponge particles were injected through the tumor-feeding branch. Selective cTACE was defined as occlusion of the segmental or subsegmental arterial feeder.

Post-procedural assessment

After completion of the procedure, all patients were admitted in the hospital for at least 72 h. During

hospitalization period, the following parameters were recorded: Symptoms (nausea, vomiting, fever, abdominal pain, and anorexia), vital signs, and other adverse events. Laboratory parameters, including liver function test, erythrocyte sedimentation rate, and C-reactive protein, were assessed at 24 and 48 h post-procedure. Hemoculture, urinalysis, complete blood count, and chest X-ray were performed if body temperature was > 38 °C.

Outcome measurement

The primary outcome was the development of PES after TACE within 48 h. In the present study, PES was identified using three different definitions: (1) SWOG toxicity coding score characterized by fever, nausea, vomiting, and/or abdominal pain within 48 h post-procedure, defined as calculated sum score more than two point (Supplementary Table 1)[7]; (2) Criteria defined by Ogasawara *et al*[11] in a randomized double-blind control study of DEXA, based on Common Terminology Criteria for Adverse Events (CTCAE) [version 5 (Supplementary Table 2) with symptoms other than those of grade I]; and (3) Criteria defined by Siramolpiwat *et al*[17] in a randomized controlled trial of single NAC dose (temperature ≥ 38.5 °C and 3-fold higher alanine transaminase level from baseline within 48 h post-procedure). The secondary outcome was the development of post-TACE liver decompensation, defined as an increase in Child-Pugh score of more than two points or newly developed decompensating events, such as ascites, hepatic encephalopathy, or serum total bilirubin > 2 mg/dL. Other cumulative adverse events (classified by CTCAE) and length of hospital stay were compared between the two groups. In patients with suspected infection or > 38 °C body temperature, laboratory testing and septic work-up were performed; moreover, these patients were treated with empirical antibiotic therapy until fever subsided or hemoculture was negative. All patients were followed up after 7 d to evaluate other post-procedure events.

Statistical analysis

Continuous data are presented as mean with standard deviation (SD) or median with range. Student's *t* test or the Mann-Whitney *U* test was performed to compare between two groups. For categorical data, Chi-square test or Fisher's exact test was applied. Statistical significance was set at $P < 0.05$. For analysis of factors that impact PES development, a logistic regression analysis was performed. Data are reported as odds ratio (OR) with 95% confidence interval (CI). All serious adverse events were reported to the Institutional Review Board of the Faculty of Medicine, Vajira Hospital, Navamindradhiraj University.

RESULTS

Patient characteristics

A total of 124 patients with HCC were screened from November 2020 to January 2022. Eighteen patients who refused to participate in this study, four patients who progressed to Child-Pugh class C, and two patients with main portal vein thrombosis were excluded. The remaining 100 patients who underwent TACE randomly received NAC-DEXA ($n = 50$) or placebo ($n = 50$) treatment. Figure 1 illustrates a flow chart of patients enrolled in this study. The mean age of patients was 60.6 years, with a male predominance (89%). The prevalence of comorbid diabetes was not different between the NAC-DEXA and placebo groups (34% *vs* 32%, $P = 0.83$). All patients exhibited cirrhosis, the majority of which with an etiology of chronic hepatitis B and alcoholic cirrhosis, followed by chronic hepatitis C, and a minority showing non-alcoholic steatohepatitis etiology. Most patients (83%) were classified as Child-Pugh class A, with no difference in mean Child-Pugh scores between the two groups (5.5 ± 0.8 in NAC-DEXA *vs* 5.5 ± 0.9 in placebo). Almost all patients (91%) were in BCLC stage B. Nine patient in BCLC stage A were justified for TACE as it would serve as a bridging therapy before curative treatment. Nearly half of the patients exhibited alpha fetoprotein (AFP) level > 200; however, the distribution of AFP level was not statically significant in both groups. Per tumor characteristics, no difference in the median tumor diameter between the two groups (NAC-DEXA, 5.5 cm; range 1.4-19 cm *vs* placebo, 7.95 cm; range 1.4-17.2 cm; $P = 0.39$) was observed. More than 50% of patients in both groups had multiple nodules (Table 1). Approximately 34% of the patients underwent their first TACE session. The type of chemotherapy and the volume of lipiodol did not differ between the two groups. Level of embolization was selected based on tumor position. In the same TACE episode, more than one-third (36%) of patients in the NAC-DEXA group had embolization of more than two branches.

Primary outcome

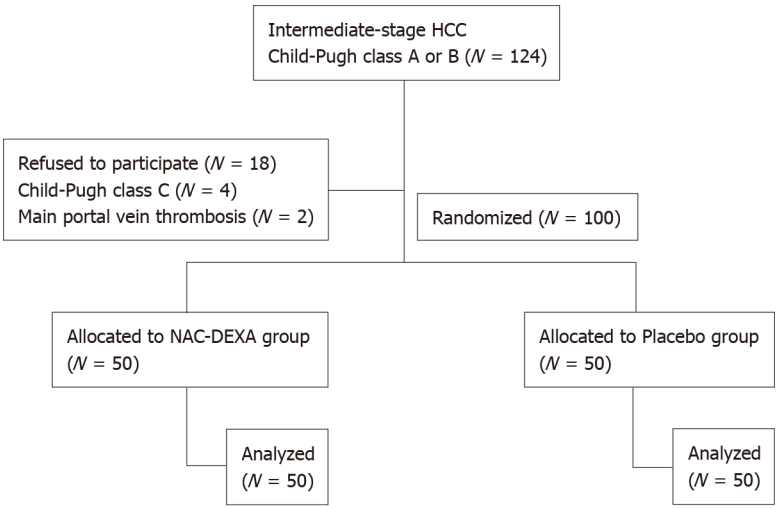
According to the various pre-defined criteria mention above, PES was detected in 43% of the patients. Most patients with PES had fever (93.0%) and nausea (72.1%), while only five (11.6%) patients had abdominal pain. The occurrence of PES after TACE was significantly lower in the NAC-DEXA group than in the placebo group in all PES-defining criteria (SWOG score more than 2 point, 6% *vs* 80%; Siramolpiwat *et al*[17] criteria, 2% *vs* 54%; and Ogasawara *et al*[11] criteria, 10 % *vs* 84 %; all $P < 0.001$) as shown in Figure 2A. The NAC-DEXA group had a lower mean SWOG PES score than the placebo group

Table 1 Baseline characteristics of patients with intermediate-stage hepatocellular carcinoma

	N-acetylcysteine + dexamethasone	Placebo	*P* value
Sex			
Male	44 (88%)	45 (90%)	0.75
Age (mean ± SD)	60.8 ± 10.52	60.46 ± 11.27	0.88
Underlying disease			
Diabetic mellitus	17 (34%)	16 (32%)	0.83
Hypertension	22 (14%)	28 (56%)	0.23
Dyslipidemia	14 (28%)	18 (36%)	0.40
CKD	1 (2%)	2 (4%)	0.56
HIV	1 (2%)	1 (2%)	1.00
Tumor characteristic			
Size			
Median (range)	5.5 (1.4-19)	7.95 (1.4-17.2)	0.39
> 3 cm	40 (80%)	42 (84%)	0.65
Number			
Median (range)	2 (1-10)	2 (1-10)	0.21
1	23 (46%)	17 (34%)	0.56
≥ 2	27 (54%)	33(66%)	
Etiology			
Hepatitis B	23 (46%)	28 (56%)	0.31
Hepatitis C	15 (30%)	12 (24%)	0.50
Alcoholic	22 (44%)	20 (40%)	0.67
Non-alcoholic steatohepatitis	3 (6%)	4 (8%)	0.70
Staging			
BCLC-A	6 (12%)	3 (6%)	0.23
BCLC-B	44 (88%)	47 (94%)	
Child-Pugh Score			
A (5-6)	40 (80%)	43 (86%)	0.42
B (7-8)	10 (20%)	7 (14%)	
ALBI score (mean ± SD)	-2.61 ± 0.58	-2.54 ± 0.53	0.54
Alpha fetoprotein (ng/mL)			
< 20	18 (36%)	19 (38%)	0.50
20-200	14 (28%)	9 (18%)	
201-1000	10 (20%)	9 (18%)	
> 1000	8 (16%)	13 (26%)	
Episode of TACE			
1	16 (32%)	18 (36%)	0.67
2-5	34 (68%)	32 (64%)	
Embolization agents			
Mitomycin (mg)			
5-10	1 (2%)	3 (6%)	0.07
10.1-15	20 (40%)	10 (20%)	

15.1-20	29 (58%)	37 (74%)	
Lipiodol (mL)			
< 5	1 (2%)	1 (2%)	0.47
5-10	13 (26%)	8 (16%)	
> 10	36 (72%)	41 (82%)	
Level of embolization			
Left hepatic artery	17 (34%)	14 (28%)	0.51
Right hepatic artery	46 (92%)	44 (88%)	0.50
Middle hepatic artery	5 (10%)	3 (6%)	0.46
≥ 2 major branches	18 (36%)	8 (16%)	0.02

ALBI: Albumin-bilirubin; BCLC: Barcelona Clinic Liver Cancer; CKD: Chronic kidney disease; HIV: Human immunodeficiency virus; SD: Standard deviation; TACE: Transarterial chemoembolization.



DOI: 10.3748/wjg.v29.i5.890 **Copyright** ©The Author(s) 2023.

Figure 1 Flow chart of patient screened, recruited, and analyzed in the study (consort diagram). HCC: Hepatocellular carcinoma; NAC: N-acetylcysteine; DEXA: Dexamethasone.

(0.38 ± 1.1 *vs* 4.04 ± 2.2; $P < 0.001$). Baseline characteristic comparison of patients with or without PES after TACE is shown in Table 2. The PES group had a higher proportion of patients with large tumor size (> 5 cm) (67.4% *vs* 47.4%; $P = 0.045$) and massive tumor burden (up to 12 criteria; 48.8% *vs* 26.3%; $P = 0.02$). Neither volume of embolizing agents nor level of vessels embolization influenced PES occurrence. Nevertheless, most (86%) patients in the PES group underwent TACE with single vessel embolization technique.

Secondary outcomes

Interestingly, post-TACE liver decompensation occurred only in the placebo group (14% *vs* 0%; $P < 0.006$). All cases were accompanied by PES. A higher proportion of patients with baseline Child-Pugh class B was observed in the post-TACE liver decompensation group, but no statistical significance was found (28.6% *vs* 16.1%, $P = 0.39$). A higher proportion of patients with abnormal liver function test, except albumin levels, was observed in the liver decompensation group. Neither tumor burden nor number of cTACE episodes influenced the occurrence of post-TACE liver decompensation. Multiple vessel embolization was performed for more patients in the post-TACE liver decompensation group compared with the group without liver decompensation (57.1% *vs* 28%, $P = 0.02$). A shorter median duration of hospital stay was observed in the NAC-DEXA group (4 *vs* 6 d; $P < 0.001$) as seen in Figure 2B. Most patients with PES were febrile, requiring empirical antibiotics therapy that was provided until negative hemoculture was obtained; this was the main reason for increased duration of hospitalization. Acute kidney injury was observed in three patients with baseline chronic kidney disease. All of them showed improved creatinine level and glomerular infiltration rate and received standard intravenous fluids at a rate of 60 mL/h from 24 h pre-TACE till 48 h post-TACE.

Table 2 Comparison between patients based on the occurrence of post-embolization syndrome and liver decompensation after conventional transarterial chemoembolization

	PES after cTACE		*P* value	Liver decompensation after TACE		*P* value
	Yes (*n* = 43)	No (*n* = 57)		Yes (*n* = 7)	No (*n* = 93)	
Male	40 (93%)	49 (86%)	0.26	7 (100%)	82 (88.2%)	0.34
Age (mean ± SD)	60.72 ± 11.11	60.56 ± 10.74	0.26	63.57 ± 7.91	60.41 ± 11.04	0.35
Child-Pugh score	5.35 ± 0.69	5.61 ± 0.9	0.09	5.86 ± 0.9	5.47 ± 0.82	0.24
A (5-6)	40 (93%)	43 (75.4%)	0.02	5 (71.4%)	78 (83.9%)	0.4
B (7-8)	3 (7%)	14 (24.6%)	0.02	2 (28.6%)	15 (16.1%)	0.4
ALBI (median; IQR)	-2.72 (-3.05, -2.29)	-2.65 (-2.93, -2.2)	0.47	-1.95 (-2.74, -1.77)	-2.69 (-3.04, -2.29)	0.09
MELD (mean ± SD)	11.67 ± 3.54	12.05 ± 3.78	0.61	12.71 ± 3.86	11.83 ± 3.66	0.54
Staging						
BCLC-A	3 (7%)	6 (10.5%)	0.54	1 (14.3%)	8 (8.6%)	0.61
BCLC-B	40 (93%)	51 (89.5%)	0.54	6 (85.7%)	85 (91.4%)	0.61
Tumor characteristics						
AFP ≥ 200 ng/mL	17 (39.5%)	27 (47.4%)	0.44	3 (42.9%)	28 (30.1%)	0.48
Median (range)	9.6 (4,13.2)	5 (3.2,10)	0.05	5.7 (3.2,15)	7 (3.2,13)	0.8
Large tumor ≥ 5 cm	29 (67.4%)	27 (47.4%)	0.045	4 (57.1%)	52 (55.9%)	0.95
Number						
1	18 (41.9%)	22 (38.6%)	0.74	4 (57.1%)	36 (38.7%)	0.34
≥ 2	25 (58.1%)	35 (61.4%)		3 (42.9%)	57 (61.3%)	
Size plus number						
Up to 7	32 (74.4%)	37 (64.9%)	0.31	4 (57.1%)	65 (69.9%)	0.48
Up to 12	21 (48.8%)	15 (26.3%)	0.02	3 (42.9%)	33 (35.5%)	0.7
cTACE episode						
1	15 (34.9%)	19 (33.3%)	0.87	4 (57.1%)	30 (32.3%)	0.18
≥ 2 (2-5)	28 (65.1%)	38 (66.7%)		3 (42.9%)	63 (67.7%)	
Embolization agent						
Mitomycin (mg)						
< 10	2 (4.7%)	2 (3.5%)	0.44	0 (0%)	4 (4.3%)	0.51
10-15	10 (23.3%)	20 (35.1%)		1 (14.3%)	29 (31.2%)	
15.1-20	31 (72.1%)	35 (61.4%)		6 (85.7%)	60 (64.5%)	
Lipiodol (mL)						
< 5	1 (2.3%)	1 (1.8%)	0.32	0 (0%)	2 (2.2%)	0.82
5-10	6 (14%)	15 (26.3%)		1 (14.3%)	20 (21.5%)	
> 10	36 (83.7%)	41 (71.9%)		6 (85.7%)	71 (76.3%)	
Level of embolization						
Left hepatic artery	10 (23.3%)	18 (31.6%)	0.36	3 (42.9%)	28 (30.1%)	0.32
Right hepatic artery	38 (88.4%)	44 (77.2%)	0.15	7 (100%)	83 (89.2%)	0.37
Middle hepatic artery	1 (2.3%)	7 (12.3%)	0.07	0 (0%)	8 (8.6%)	0.43
1 vessel	37 (86%)	34 (59.6%)	0.004	3 (42.9%)	67 (72%)	0.25
≥ 2 vessels	6 (14%)	23 (40.4%)	0.007	4 (57.1%)	26(28%)	0.02
Baseline laboratory						

(median; IQR)						
Hct (%)	36.8 (32.3, 40.3)	35 (32.6, 38.9)	0.33	39.8 (36.6, 40.6)	35.2 (32.2, 39)	0.04
WBC (× 10^9/L)	5.83 (4.50, 7.18)	5.48 (4.13, 6.97)	0.18	5.49 (4.14, 6.08)	5.58 (4.29, 7.07)	0.57
Platelet (× 10^9/L)	186 (125, 241)	144 (103, 204)	0.09	105 (65, 226)	171 (117, 230)	0.39
PTT (s)	12.9 (12.3, 13.8)	13.5 (12.7, 14.7)	0.02	13 (12.1, 13.2)	13.1 (12.4, 14.2)	0.34
AST (U/L)	51 (35, 102)	47 (37, 79)	0.78	163 (102, 169)	49 (36, 77)	0.003
ALT (U/L)	35 (20, 80)	33 (21, 51)	0.29	106 (80, 139)	31 (20, 51)	< 0.001
ALP (U/L)	124 (104, 290)	120 (93, 153)	0.211	290 (121, 386)	120 (94, 169)	0.111
Albumin (g/dL)	4 (3.6, 4.2)	3.9 (3.5, 4.2)	0.569	3.7 (3.2, 4.1)	3.9 (3.6, 4.2)	0.360
Total bilirubin (mg/dL)	0.59 (0.39, 1)	0.69 (0.51, 1.3)	0.163	1.59 (0.82, 2.11)	0.59 (0.42, 1.01)	0.005
Direct bilirubin (mg/dL)	0.35 (0.24, 0.65)	0.39 (0.26, 0.78)	0.415	0.91 (0.49, 1.76)	0.35 (0.24, 0.65)	0.004
Presence of PES, *n* (%)	-	-	-	7 (100%)	36 (38.7%)	0.002
Fever	40 (93.0%)	5 (8.8%)	< 0.001	7 (100%)	38 (40.9%)	0.002
Vomit	25 (58.1%)	2 (3.5%)	< 0.001	3 (42.9%)	24 (25.8%)	0.327
Pain	5 (11.6%)	0 (0.0%)	0.008	0 (0.0%)	5 (5.37%)	0.529
Anorexia	21 (48.8%)	2 (3.5%)	< 0.001	4 (57.1%)	17 (18.3%)	0.015

AFP: Alpha fetoprotein; ALBI: Albumin-bilirubin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BCLC: Barcelona Clinic Liver Cancer; cTACE: Conventional transarterial chemoembolization; Hct: Hematocrit; IQR: Interquartile range; MELD: Model of end-stage liver disease; PES: Post-embolization syndrome; PTT: Prothrombin time; SD: Standard deviation; WBC: White blood cell.

Predictors of PES

In univariate analysis of tumor volume, calculated increase in Albumin-Bilirubin (ALBI) score of more than 0.5 and a dynamic change in liver function (more than 20-fold and 1.5-fold increase from baseline in transaminase and bilirubin levels, respectively) within 48 h post-TACE were associated with the development of PES. Multivariate analysis showed that only intravenous NAC-DEXA pre-procedure could reduce the incidence of PES (OR = 0.04; 95%CI: 0.01-0.2; P < 0.001) (Table 3).

Predictors of liver decompensation

In univariate analysis, an occurrence of PES after TACE with an SWOG score > 4, more than 0.5 point increase in ALBI score, and a 1.5-fold increase from baseline in bilirubin level were associated with the development of liver decompensation (Table 3). In multivariate analysis, only a dynamic change in ALBI score > 0.5 point was considered an important risk for occurrence of liver decompensation with an OR of 42.77 (95%CI: 1.01-1810; P = 0.049).

Safety

Only two patients in the NAC-DEXA group developed a minor allergic skin reaction; in which, we disrupted treatment immediately. Six hours after drug discontinuation and administration of anti-allergic medication, the symptoms resolved. Subsequently, both patients completed the NAC-DEXA protocol with a lower infusion rate. No serious adverse event was reported in the NAC-DEXA group (Figure 2C). One patient in the placebo group died within 90 d post-procedure due to severe sepsis with liver decompensation. Although most patients were febrile post-TACE procedure, none contracted intrahospital secondary bacterial infection. The incidence of hyperglycemia did not differ between the NAC-DEXA and placebo groups (34% *vs* 32%, P = 0.83). Only three patients experienced grade 3 CTCAE hyperglycemia that was managed with antidiabetic therapy. According to changes in the liver function test at 48 h post-TACE, the 3-fold increase in total bilirubin level from baseline, but not transaminase, was more pronounced in the placebo group compared with that in the NAC-DEXA group (58 % *vs* 18 %, P = 0.006; Supplementary Table 3).

DISCUSSION

Pathogenesis of PES is conceivably related to multiple factors, such as direct toxic effects of chemotherapeutic agents and release of inflammatory cytokines related to tumor cell necrosis or ischemic hypoxic injury of normal hepatocytes[13,17,20]. The incidence of PES after TACE, per previous reports, ranges

Table 3 Univariate and multivariate analyses of patients with post-embolization syndrome and liver decompensation after conventional transarterial chemoembolization

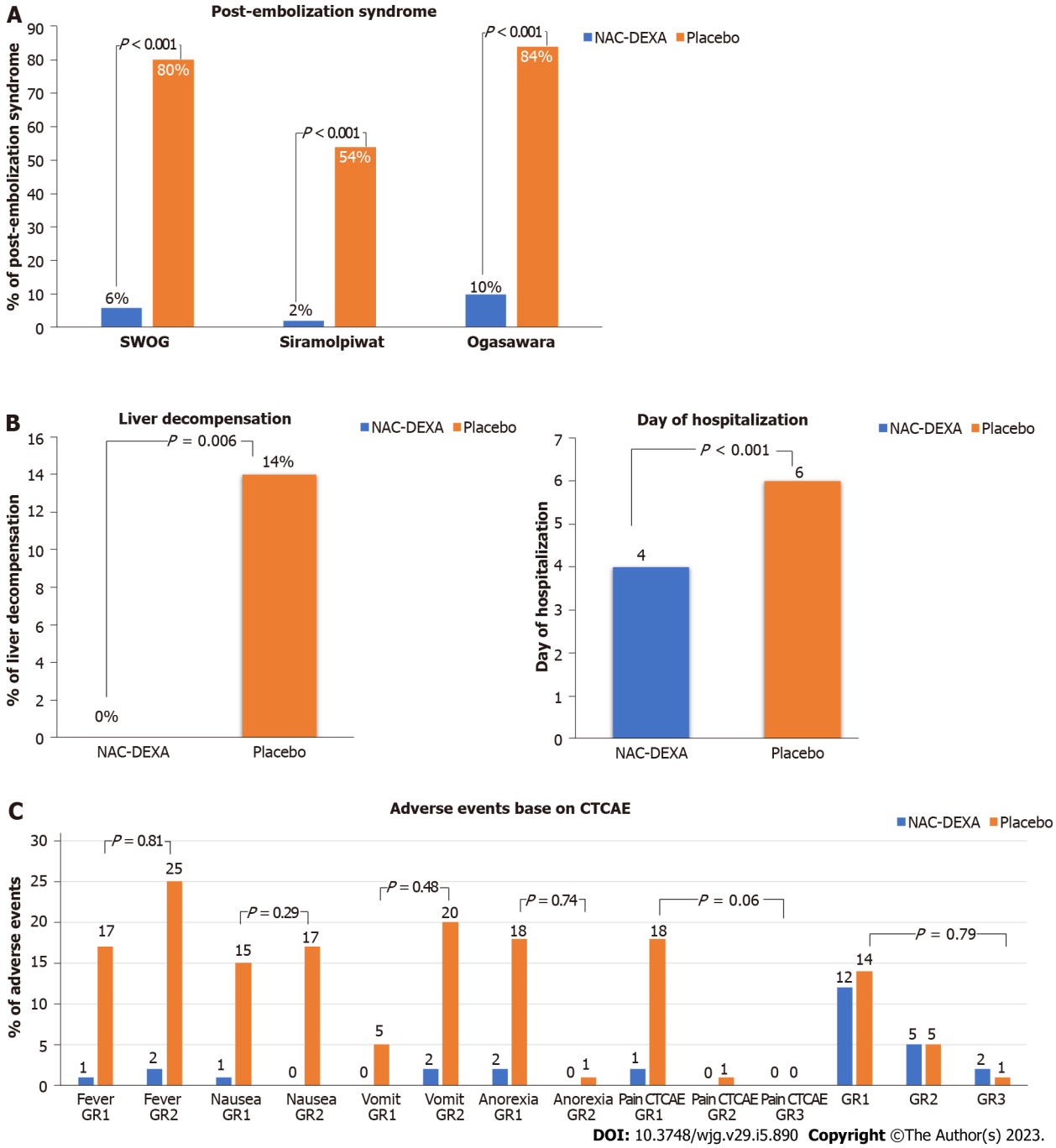
Parameter	PES after cTACE						Liver decompensation after cTACE					
	Crude OR	95%CI	*P* value	aOR	95%CI	*P* value	Crude OR	95%CI	*P* value	aOR	95%CI	*P* value
NAC + DEXA	0.02	0-0.06	< 0.01	0.04	0.01-0.2	< 0.01	0	0-1	1.00	0	0-1	1.00
Size + number up to 12	1.09	1-1.19	0.04	1.08	0.92-1.27	0.33	0.99	0.84-1.16	0.88	-	-	-
AST rise > 20 folds in 48 h	1.51	1.09-2.09	0.01	1.35	0.72-2.52	0.35	1.7	0.95-3.03	0.07	1.3	0.56-3.02	0.54
ALBI change > 0.5	7.58	1.96-29.36	0.003	3.03	0.39-23.67	0.29	122.52	5.6-2681	0.002	42.77	1.01-1810	0.049
Total bilirubin rising > 1.5 folds	3.83	1.8-8.14	< 0.01	1.43	0.42-4.87	0.57	2.79	1.23-6.31	0.01	1.46	0.52-4.1	0.48
South west oncology grading > 4	1	0-1	0.99	-	-	-	1.43	1.04-1.96	0.03	0.94	0.51-1.76	0.86

ALBI: Albumin-bilirubin; aOR: Adjusted odds ratio; AST: Aspartate aminotransferase; CI: Confidence interval; DEXA: Dexamethasone; NAC: N-acetylcysteine; OR: Odds ratio; PES: Post-embolization syndrome; TACE: Transarterial chemoembolization.

widely (45%-83%). Studies that did not provide a strategic prophylaxis for PES reported an incidence of up to 80%. The incidence of PES in the present study was 43%. PES was verified by the occurrence of symptoms such as fever, nausea, vomiting, and abdominal pain, using SWOG toxicity code score of more than two point[7]. Fever is the most common symptom exhibiting a heterogeneity of prevalence (11.6%-74%), followed by nausea (11.6%-80.6%) and vomiting (16.2%-58.9%)[6,9,12,13,21]; in this study, the reported prevalence is 90%, 66%, and 54% for fever, nausea, and vomiting, respectively. Amelioration of nausea and vomiting by ondansetron premedication (8 mg) and selection of the less adverse chemotherapeutic events-causing agent, mitomycin C, may explain the lower incidence of nausea/vomiting in the present study than that in previous studies. Despite the application of super-selective single vessel embolization technique by radio-intervention at our center, a large tumor burden and a trend of higher mitomycin dose (> 10 mg) was observed and may have affected both tumor and normal liver cell necrosis, resulting in a higher PES occurrence.

NAC not only lowers free-radical levels, attributed to its antioxidant properties, but also acts as an indirect antioxidant by increasing the glutathione level and anti-inflammatory effect[22]. Therefore, many gastrointestinal guidelines recommend NAC for the treatment of alcoholic hepatitis[23], acetaminophen overdose[24], and non-acetaminophen acute liver failure[25]. Other favorable effects could be linked to its hepatoprotective activity. Interestingly, Siramolpiwat *et al*[17] demonstrated this protective effect; intravenous NAC minimized PES compared with the placebo group even though criteria for PES diagnosis was defined as only occurrence of fever and elevated serum alanine transaminase, without reference to clinical symptoms.

The beneficial effects of DEXA were presumably attributed to its antiemetic and inflammation dampening properties. Kogut *et al*[26] demonstrated that patients receiving prophylactic DEXA tended to require lower doses of antiemetic agents than those who do not. Ogasawara *et al*[11] reported that intravenous administration of a combination of DEXA and antiemetics (total 36 mg) for 3 d ameliorated PES by 52.5%. Recently, a randomized controlled trial by Sainamthip *et al*[12] demonstrated that a single



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Figure 2 Post-embolization syndrome, liver decompensation and length of hospital stay and adverse events after conventional transarterial chemoembolization. A: Post-embolization syndrome after conventional transarterial chemoembolization; B: Post-conventional transarterial chemoembolization liver decompensation and length of hospital stay; C: Post-conventional transarterial chemoembolization adverse events. NAC: N-acetylcysteine; DEXA: Dexamethasone.

dose of intravenous DEXA (8 mg) can prevent PES, achieving a negative PES rate of 63.3%.

We decided to maximize the protective effect of DEXA and NAC in prevention of PES by combining the two, with intravenous administration of DEXA (cumulative dose of 36 mg) and NAC 24 h before and continuous infusion until 48 h after cTACE. Interestingly, our study found that pre-TACE therapy with NAC-DEXA regimen led to a lower PES occurrence of less than 10%, which is the lowest incidence compared with those reported in previous publications (24.6% in a NAC study[17] and 37%-47% in DEXA studies[11,12]. The synergistic effect of NAC and DEXA can diminish systemic inflammatory response and ischemic hepatitis[27-29]. Hence, this study emphasizes the advantage of NAC-DEXA combination due to its synergistic PES-reducing effect.

Post-TACE liver decompensation is one of the most important complications and is associated with significant morbidity and mortality[21,30]. It is characterized by an increase in Child-Pugh score of more than 2 points, ≥ 2 mg/dL rise in serum total bilirubin level, newly developed ascites, or hepatic encephalopathy within two weeks post-procedure. In the present study, seven patients developed post-TACE

liver decompensation. Previous studies reported that portal vein thrombosis, poor baseline liver status, high serum AFP, and PES were associated with this complication[17,27,28]. Correspondingly, all patients with liver decompensation in our study had concurrent PES. In patients who underwent post-TACE without any prophylaxis treatment, we observed a lower incidence (14%) of liver decompensation compared with those in previous reports (13.4%-17.3%)[28,29]; the lower incidence in our study is attributed to the fact that a majority of patients had good liver reserve with Child-Pugh class A. Because all eligible cTACE patients in the present study fulfilled BCLC staging criteria, neither Child-Pugh nor Model for End-Stage Liver Disease score influenced the outcome. Further, more than half of our decompensated patients received cTACE intervention with multiple vessel embolization technique, which may have compromised the vessels; however, owing to the small sample size in this group, further studies are warranted.

ALBI score is the only objective parameter to stratify patients into different grades and is used to predict the prognosis of all HCC stages. This new model outperforms the Child-Pugh score for evaluation of liver function reserve. In a previous study, evaluation of baseline ALBI score in HCC patients who underwent cTACE not only predicted survival but also estimated liver decompensation and liver failure[31]. In our study, the mean pre-treatment ALBI score was not significantly different between the NAC-DEXA and placebo groups. Our findings indicate that the proportion of preserved liver, defined by ALBI grade 1, was comparable between the two groups. Moreover, all patients who developed post-TACE liver decompensation had a baseline ALBI grade 1 or 2 with a mean pre-treatment ALBI score of -2.57. The increase in ALBI score was hypothesized to be a novel non-invasive tool for earlier prediction of post-TACE liver decompensation than Child-Pugh score. Our study demonstrated that a dynamic increase in ALBI score of more than 0.5 point had a marked impact on liver decompensation. Thus, the application of dynamic increase in ALBI score, but not albumin level, for a prediction of early post-TACE liver decompensation requires further research.

Limitation

Our study had some limitations. First, we did not perform dose optimization for DEXA and NAC in order to minimize PES. Second, this dual treatment was not compared with single DEXA. The dynamic changes in cytokine levels due to this dual treatment is an interesting topic for further study.

CONCLUSION

A combination of DEXA and NAC can not only maximize the reduction in PES incidence but also shorten hospitalization period in HCC patients undergoing cTACE procedure. Dynamic alterations in ALBI score, but not CPS, may predict liver decompensation.

ARTICLE HIGHLIGHTS

Research background

Transarterial chemoembolization (TACE) is the current standard treatment for intermediate-stage hepatocellular carcinoma (HCC). Post-embolization syndrome (PES) is a complex clinical syndrome which may occur after conventional TACE (cTACE). Either N-acetylcysteine (NAC) or dexamethasone (DEXA) is used to prevent PES.

Research motivation

The synergistic effect of the combined therapy for preventing PES and liver decompensation has not been determined.

Research objectives

The aim of this study was to evaluate the efficacy of NAC and DEXA combination in preventing PES and liver decompensation after cTACE.

Research methods

A single-center randomized controlled clinical trial.

Research results

Our study provides clinical evidence that intravenous NAC plus DEXA administration ameliorates the occurrence of post-TACE PES in patients with intermediate-stage HCC. Interestingly, we found that a dynamic change in Albumin-Bilirubin (ALBI) score was a risk factor for post-TACE liver decompensation.

Research conclusions

A combination of NAC and DEXA ameliorated the occurrence of PES after cTACE in patients with intermediate-stage HCC.

Research perspectives

The application of dynamic increase in ALBI score for a prediction of early post-TACE liver decompensation requires further research.

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FOOTNOTES

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Country/Territory of origin: Thailand

ORCID number: Nitipon Simasingha 0000-0002-7883-3294; Wasu Tanasoontrarat 0000-0003-1698-4465; Torpong Claimon 0000-0002-6613-2650; Supatsri Sethasine 0000-0002-7637-3669.

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LETTER TO THE EDITOR

Timing of biliary decompression for acute cholangitis

Jian Yang, Ying Liu, Shi Liu

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Jian Yang, Shi Liu, Central Laboratory, The Third Affiliated Hospital, Qiqihar Medical University, Qiqihar 161000, Heilongjiang Province, China

Ying Liu, Department of Medical Oncology, The Third Affiliated Hospital, Qiqihar Medical University, Qiqihar 161000, Heilongjiang Province, China

Corresponding author: Shi Liu, PhD, Chief Physician, Central Laboratory, The Third Affiliated Hospital, Qiqihar Medical University, No. 27 Taishun Street, Tiefeng, Qiqihar 161000, Heilongjiang Province, China. shiliu2199@163.com

Abstract

Severe acute cholangitis (AC) exacerbates the risk of death because of the rapid progression of the disease. The optimal timing of biliary decompression (BD) as a necessary intervention in patients with severe AC is controversial. A recently report titled "Timing of endoscopic retrograde cholangiopancreatography in the treatment of acute cholangitis of different severity" in the *World Journal of Gastroenterology* that the optimal time of endoscopic retrograde cholangiopancreatography for treating patients with severe AC is ≤ 48 but not ≤ 24 h, providing clinical evidence for selecting the optimal time for implementation of BD. Here, we discuss the controversy over the optimal timing of BD for AC based on guidelines and clinical evidence, and consider that more high-level clinical researches are urgent needed to benefit the management of patients with different severity of AC.

Key Words: Acute cholangitis; Biliary decompression; Endoscopic retrograde cholangiopancreatography; Severity; Optimal time; Clinical evidence

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Core Tip: Severe acute cholangitis (AC) exacerbates the risk of death because of the rapid progression of the disease. The optimal timing of biliary decompression (BD) as an intervention for severe AC is controversial. The purpose of this letter is to highlight the controversy surrounding the existing clinical evidence regarding BD for the treatment of AC of varying severity and to suggest that clinical studies providing higher levels of evidence will improve the therapeutic benefits.

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TO THE EDITOR

Acute cholangitis (AC) originates as an infection of the extrahepatic biliary system and is usually characterized by rapid progression leading to systemic sepsis. The morality rate of severe AC may reach 30% [1]. In most cases, biliary decompression (BD) is necessary to treat patients with severe AC, regardless of the treatment modality chosen [endoscopic retrograde cholangiopancreatography (ERCP), percutaneous transhepatic biliary drainage, or stone removal][2]. However, the optimal time at which to implement BD to obtain the maximum therapeutic benefit for patients with AC remains uncertain.

We recently became extremely interested in a retrospective study by Huang *et al*[3] published in the October 2022 issue of the *World Journal of Gastroenterology*. This was a high-quality observational study with a Newcastle-Ottawa Quality Assessment Scale score of 7 (3, 2, 2)[4]. It was independently assessed by two of our authors, and disagreements were resolved by a third author. Based on a retrospective analysis of 683 patients with AC, the conclusions drawn by the authors properly summarize the data in the study. The authors' data indicated that 30-d mortality in patients with AC was not significantly different between ERCP performed at > 24 and ≤ 24 h. However, patients with AC had lower 30-d mortality and a shorter length of stay when ERCP was performed at ≤ 48 h. Additionally, patients with grade III AC had lower 30-d mortality rates than patients with grade I and II AC, although they had higher intensive care unit admission rates and longer lengths of stay[3]. Huang *et al*[3] suggested that a ≤ 48-h duration from the patient's presentation to initiation of ERCP therapy, rather than a ≤ 24-h duration, provided the best survival benefit for patients with AC, especially for patients with grade III AC. This unique insight breaks with the traditional treatment concept that earlier performance of BD is associated with better outcomes in patients with severe AC. We thank Huang *et al*[3] for their study, which provides clinical evidence for the optimal timing of BD in patients with grade III AC.

The 2018 revised Tokyo guideline (TG18) currently serves as the most influential guideline for assessing AC severity. It delineates three grades of assessment of AC severity and indicates that the impact of the BD implementation time on the therapeutic benefit is significantly correlated with AC severity[5]. Therefore, we consider that the AC severity characterizes the urgency of the BD implementation time.

Reference Citation Analysis (RCA) (https://www.referencecitationanalysis.com/) is an artificial intelligence technology-based open multidisciplinary citation analysis database. We searched the RCA database for articles in cutting-edge fields in the last 3 years using the search terms "endoscopic retrograde cholangiopancreatography", "biliary decompression", and "acute cholangitis". Recent guidelines and clinical evidence suggest that the optimal timing of BD implementation remains controversial. First, controversy regarding the timing of BD implementation in the guideline exists primarily for patients with grade II and III AC. The American Society for Gastrointestinal Endoscopy (ASGE) 2021 guideline states that important outcome indicators for evaluating the survival benefit in patients with AC are 30-d mortality, inpatient mortality, length of stay, and organ failure[2]. The guideline also suggests that performance of ERCP at ≤ 48 h may be associated with lower 30-d mortality and a shorter length of stay[2]. However, the ASGE 2021 guideline does not report the correlation between the time to BD implementation and AC severity because the available clinical evidence is insufficient[2]. TG18 states that BD in patients with grade II AC should be performed within 24 h, and although clinical data do not indicate the optimal time for BD in patients with grade III AC, urgent decompression (within 24 h) may improve the prognosis of patients with grade III AC[5]. The European Society of Gastrointestinal Endoscopy 2019 guideline states that for patients with severe AC (grade III), implementation of BD is recommended within 12 h; for patients with moderate AC (grade II), it should be performed within 48 to 72 h[6]. Second, recent clinical studies have produced controversial results in terms of early implementation of BD for patients with grade III AC. In addition to the study by Huang *et al*[3] discussed herein (BD at ≤ 48 but not ≤ 24 h), another retrospective study by Lu *et al*[7] showed that BD is recommended at 24 h of admission for patients with grade III AC and within 12 h for those with AC accompanied by neurological or cardiovascular dysfunction. However, a study by Becq *et al*[8] using a propensity score matching approach indicated that the use of BD within 6h or 12 h of AC onset was not associated with better clinical outcomes, but possibly reduced readmission rates. Finally, the results of three recent systematic reviews and meta-analyses suggest that the controversy over the optimal timing of BD is mainly focused within 24 h and 48 h based on data analysis of superior outcomes within their respective time zones[9-11]. However, the three studies did not report that the optimal timing of BD affects survival outcomes in populations with different severities of AC (*i.e.*, grades I, II, and III)[9-11].

Based on the above-mentioned current controversies, we present the following future outlook. First, because of the controversy in the current guidelines and among recent clinical studies regarding the optimal timing of BD for patients with grade II and III AC, a multicenter prospective cohort study or randomized controlled trial should be conducted. Second, the medical community is called upon to pay attention to the clinical studies that have been reported and to perform systematic reviews and meta-analyses on the optimal time to implement BD for the treatment of patients with grade II and III AC.

In conclusion, a higher level of clinical evidence regarding the optimal time to implement BD in patients with different severities of AC is needed to improve the therapeutic benefit.

FOOTNOTES

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Country/Territory of origin: China

ORCID number: Jian Yang 0000-0002-5282-1383; Ying Liu 0000-0002-7461-0691; Shi Liu 0000-0001-6959-6904.

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Editorial Board Member of *World Journal of Gastroenterology*, Neil Donald Merrett, MBBS, FRACS, Professor, Division of Surgery, Western Sydney University, Locked Bag 1797, Penrith NSW 2571, Australia. n.merrett@westernsydney.edu.au

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OPINION REVIEW

COVID-19 and liver injury in individuals with obesity

Ioannis G Lempesis, Eleni Karlafti, Petros Papalexis, George Fotakopoulos, Kyriakos Tarantinos, Vasileios Lekakis, Stavros P Papadakos, Evangelos Cholongitas, Vasiliki E Georgakopoulou

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Ioannis G Lempesis, Institute of Metabolism and Systems Research, University of Birmingham, Birmingham, B15 2TT, United Kingdom

Ioannis G Lempesis, Department of Human Biology, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Centre, Maastricht 616 6200, Netherlands

Eleni Karlafti, Department of Emergency, AHEPA University Hospital, Aristotle University of Thessaloniki, Thessaloniki 546 21, Greece

Petros Papalexis, Unit of Endocrinology, First Department of Internal Medicine, Laiko General Hospital, National and Kapodistrian University of Athens, Athens 11527, Greece

Petros Papalexis, Department of Biomedical Sciences, University of West Attica, Athens 12243, Greece

George Fotakopoulos, Department of Neurosurgery, General University Hospital of Larisa, Larisa 41221, Greece

Kyriakos Tarantinos, First Pulmonology Department, Sismanogleio Hospital, Athens 15126, Greece

Vasileios Lekakis, Stavros P Papadakos, Department of Gastroenterology, Laiko General Hospital, National and Kapodistrian University of Athens, Athens 11527, Greece

Evangelos Cholongitas, First Department of Internal Medicine, Laiko General Hospital, Medical School, National and Kapodistrian University of Athens, Athens 11527, Greece

Vasiliki E Georgakopoulou, Department of Infectious Diseases-COVID-19 Unit, Laiko General Hospital, Athens 11527, Greece

Corresponding author: Vasiliki E Georgakopoulou, Doctor, MSc, Academic Research, Doctor, Department of Infectious Diseases-COVID-19 Unit, Laiko General Hospital, 17 Agiou Thoma Street, Athens 11527, Greece. vaso_georgakopoulou@hotmail.com

Abstract

Coronavirus disease 2019 is an infectious disease caused by the severe acute respiratory syndrome coronavirus 2 that manifests as a variety of clinical manifestations, including liver damage commonly detected by a hepatocellular pattern from liver function tests. Liver injury is associated with a worse prognosis overall. Conditions associated with the severity of the disease include obesity and

cardiometabolic comorbidities, which are also associated with nonalcoholic fatty liver disease (NAFLD). The presence of NAFLD, similarly to obesity, is associated with an unfavourable impact on the coronavirus disease 2019 outcome. Individuals with these conditions could present with liver damage and elevated liver function tests due to direct viral cytotoxicity, systemic inflammation, ischemic or hypoxic liver damage or drug side effects. However, liver damage in the setting of NAFLD could also be attributed to a pre-existing chronic low-grade inflammation associated with surplus and dysfunctional adipose tissue in these individuals. Here we investigate the hypothesis that a pre-existing inflammatory status is exacerbated after severe acute respiratory syndrome coronavirus 2 infection, which embodies a second hit to the underestimated liver damage.

Key Words: COVID-19; SARS-CoV-2; Non-alcoholic fatty liver disease; Obesity; Inflammation; Liver; Adipose tissue

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Core Tip: The severe acute respiratory syndrome coronavirus 2 that causes coronavirus disease 2019 has a variety of clinical manifestations, including liver damage. Obesity and other dysmetabolic diseases linked to nonalcoholic fatty liver disease are a few of the factors that contribute to the severity of the illness. Due to direct viral cytotoxicity, people with these illnesses may have liver damage and increased liver function tests. However, liver injury might also be related to pre-existing inflammation and the detrimental effects of excessive and dysfunctional adipose tissue in these people.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)[1,2], which was first identified in December 2019 in Wuhan, China, and has since spread globally, resulting in an ongoing pandemic[2]. COVID-19 represents a systemic disease that can cause a variety of clinical manifestations, which range from asymptomatic individuals to mild respiratory symptoms and severe critical illness[3-7]. A plethora of underlying pathways drive the pathophysiology, including activation or dysregulation of localized (*e.g.*, vascular) and generalized inflammation, which leads to multiorgan failure and eventually death[8-12].

Among the clinical manifestations, liver involvement, indicated by abnormal liver function tests (LFT) in patients, was noted early in the pandemic's development[13]. However, major liver damage is not common[14-18]. The liver may be affected by SARS-CoV-2 directly (*i.e.* translocation of the virus from the intestines to the liver) or *via* indirect ways (*i.e.* systemic inflammatory response, ischemic and hypoxic liver damage, drug-related liver injury and impacts on pre-existing liver diseases)[14,16,17]. Coexisting liver disease, including metabolic/obesity related nonalcoholic fatty liver disease (NAFLD), may also negatively impact the course of COVID-19[14,15].

Early observations of COVID-19 have highlighted obesity as one of the most common underlying conditions in patients hospitalized with COVID-19[19-21]. Obesity was further associated with hospital admission and as an independent predictor of severity, intensive medical intervention and mortality of COVID-19[15,19]. Obesity, determined by increased total fat mass (body mass index > 30 kg/m^2), is a chronic multifactorial disease that predisposes patients to numerous comorbidities, including metabolic diseases (insulin resistance states, type 2 diabetes mellitus, NAFLD), cardiovascular diseases[15,22-24] and various types of cancer[25-28].

Patients with poorer clinical outcomes for COVID-19 are characteristically older (more than 60 years) and have several metabolic syndrome manifestations, obesity and type 2 diabetes mellitus, a profile similar to those at increased risk of NAFLD[13,29,30]. Coexisting hepatic diseases like NAFLD and metabolic dysregulations could theoretically impact the manifestations and progression of COVID-19, and the liver injury in that population could represent a combination of the underlying condition exacerbated by the virus and inflammatory responses.

Here, we presented the current evidence for hepatic involvement in COVID-19 in obese individuals and the impact of fatty liver disease. Then, we tested the idea that liver damage in people with obesity and/or NAFLD could be caused by a "second hit" from the virus on top of hepatic dysfunction that was already there. This is likely caused by metabolic dysregulation and a chronic inflammatory state that is linked to obesity.

CURRENT EVIDENCE OF HEPATIC INVOLVEMENT IN COVID-19

Patients with COVID-19 have experienced varying degrees of liver damage, ranging from self-limited hepatitis to significant liver injury and liver failure[31,32]. Impaired LFT without other hepatic manifestations was shown to be present in almost 50% of patients with COVID-19 in various studies[31-33]. The spectrum of pathological LFT includes elevated liver enzyme concentrations of transaminases [aspartate transaminase (AST), alanine transaminase (ALT)], cholestatic enzymes including alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT), lactate dehydrogenase and bilirubin and impaired synthetic activity as seen with prothrombin time (PT) prolongation and decreased albumin concentrations[31,34]. Based on the pattern of the affected LFT, liver damage is categorized as parenchymal (AST, ALT), cholestatic (ALP, GGT) or mixed. The most common type of liver damage, in up to 75% of cases, is parenchymal, indicating hepatocellular damage as a major part of physiopathology[31,34,35].

Several studies have shown elevated AST or ALT during acute COVID-19 illness[36-39]. A higher proportion of patients with severe cases (46.2% *vs* 12.7% in mild cases) had elevated AST and ALT[36,40-42]. AST appeared to be elevated first, followed by ALT in patients with severe disease[33,34]. Accordingly, AST was found to be correlated with mortality and liver dysfunction[5], showing an increased odds ratio in severe cases in other studies[42,43]. Elevated LFT was also recorded in subclinical cases (AST in 8.7% and ALT in 8.9%)[39].

The cholestatic pattern of elevated LFT was typically accompanied by elevated parenchymal enzymes [31]. In both mild and severe cases, ALP and bilirubin levels were found to be moderately elevated[37]. In a systematic review and meta-analysis of 64 studies, it was shown that the prevalence of elevated total bilirubin (9.7%), GGT (15.0%) and ALP (4.0%) were lower compared to elevated levels of aminotransferases (AST 23.2% and ALT 21.1%)[44]. Interestingly, in a retrospective study where ALP and GGT concentration levels were usually within normal values, pathological levels of GGT were found in 37.6% of patients with NAFLD[45].

In addition, a meta-analysis of 24 studies showed that a significantly longer PT in patients with severe COVID-19 was associated with higher morbidity rates. Elshazli *et al*[46] also concluded in their systematic review that prolonged PT was associated with a higher risk of disease progression to severe COVID-19 and admission to an intensive care unit. Impaired synthetic liver function, as assessed by hypoalbuminemia, was present and associated with an increased risk in patients with COVID-19[47]. Similarly, hypoalbuminemia was correlated with the severity of the disease and morbidity[31].

Overall, irrespectively of which LFT was affected, once hepatic damage was involved, patients had an increased risk of mortality and severe disease, which was shown in a systematic review of 107 studies and 20874 patients[48]. Acute liver injury is an important predictor for disease development[49]. In patients with chronic liver disease, including NAFLD, liver damage may occur more often. Chronic liver disease from any cause was discovered to be a risk factor for extended hospitalization and the fatal course of COVID-19[31].

FATTY LIVER DISEASE AND COVID-19

The occurrence of NAFLD in patients with SARS-CoV-2 infection is substantial[4,31,50]. This is the case as specific cardiometabolic chronic conditions intrinsically linked to NAFLD (hypertension, diabetes, obesity, coronary artery disease, and cerebrovascular disease) have been noted as important risk factors associated with an increased risk of severe COVID-19[4,45,51]. In a recent meta-analysis and systematic review of 16 studies, Hayat *et al*[29] examined NAFLD prevalence, COVID-19 and the outcomes among patients with NAFLD *vs* those without. The study findings showed that individuals with NAFLD, as compared to those without, had a higher probability of severe COVID-19 and intensive care unit admission[29]. Furthermore, NAFLD patients tended to have higher COVID-19-related mortality compared to non-NAFLD patients[6,29].

Assessment of LFT and clinical outcomes in patients with or without NAFLD showed that in the vast majority of patients, liver damage was minor and hepatocellular in type[13,45]. A higher probability of abnormal LFT from admission to discharge was recorded for patients with NAFLD[13,45]. A Chinese analysis found that patients with NAFLD had a higher incidence of severe COVID-19 than those who did not have NAFLD but only those under 60-years-old. This observation was observed even after adjusting for potentially confounding factors like obesity, diabetes and hypertension[13,52]. In contrast to that age group, there was no association between patients age 60 years and older and NAFLD and the severity of COVID-19[13,52]. It was suggested that NAFLD-induced hepatic and systemic immune

responses may worsen the severity of the cytokine storm in COVID-19 patients who are younger. Aside from that, older people often have other cardiometabolic comorbidities, which may change any link to NAFLD[13,52].

NAFLD AND OBESITY

Dysfunctional adipose tissue (AT), an organ critical for whole-body homeostasis, is a hallmark of obesity and obesity-related chronic cardiometabolic diseases[28,53-55]. Dysfunctional AT is distinguished by, among other things, hormonal/adipocytokine dysregulation, a state of chronic low-grade inflammation associated with adipocyte hypertrophy and lipid metabolism impairments (reduced capacity to buffer the daily influx of dietary lipids), resulting in ectopic fat accumulation[28,56-58]. Additionally, decreased AT blood flow, mitochondrial dysfunction and altered oxygenation are frequently present in obesity[28,59].

Several proinflammatory molecules are increased in obesity and are related to aspects of the metabolic syndrome and other metabolic dysfunctions, including tumour necrosis factor alpha, interleukin 6 (IL-6), plasminogen activator inhibitor-1, dipeptidyl peptidase 4 and monocyte chemoattractant protein-1[53,54,60-62]. IL-6 can activate macrophages and contribute to the cytokine storm seen in COVID-19[13,63]. The loss of muscle mass and its anti-inflammatory properties, which are common in sarcopenic obesity, may have a negative impact on the chronic inflammatory status, various comorbidities and COVID-19 outcomes[64].

Liver dysfunction associated with obesity and metabolic diseases, including the spectrum of NAFLD, is characterized by a multifactorial pathophysiological mechanism still under investigation involving multiple pathways[65]. It has been suggested that, in part, the inability to sufficiently increase subcutaneous AT triglyceride stores in response to excessive energy intake and weight gain causes lipids to be diverted to other organs, such as the liver and skeletal muscle, where they accumulate ectopically and cause cellular lipotoxicity. This results in insulin resistance and inflammation in these tissues[66-70].

COVID-19: AN ADDITIONAL CAUSE OF LIVER DAMAGE IN PRE-EXISTING NAFLD AND OBESITY?

The COVID-19 systemic inflammatory state is linked with the angiotensin converting enzyme 2 (ACE2) receptor expressed in a variety of tissues[2,9,11]. Many organ systems, including the lungs, liver, heart, kidney and blood vessels, contain ACE2 receptors[31]. AT of patients with obesity is estimated to exhibit higher amounts of ACE2, perhaps acting as a SARS-CoV-2 reservoir with delayed viral shedding time[13,63]. The structural spike protein, characteristic of SARS-CoV-2, predominantly promotes the host's immunological reaction[9]. The spike protein attaches to ACE2 receptor sites on the cell membrane, facilitating cell penetration[9].

In the liver, ACE2 receptor expression is significantly higher in cholangiocytes than in hepatocytes[31,41]. Direct virus cytopathogenic effects, inflammatory responses and intrahepatic immune cell stimulation, (micro)vascular thrombosis, hepatic obstruction, destabilized gut-liver axis, drug-induced toxicity and interfaces all play a part in liver damage during COVID-19 infection[31,35,71]. Interestingly, as demonstrated in a diet-induced nonalcoholic steatohepatitis (NASH) model in rodents, ACE2 hepatic expression was shown to be dramatically elevated, potentially enhancing hepatic vulnerability towards SARS-CoV-2 infection in individuals with NAFLD and/or NASH[13,72]. However, the link between NAFLD and liver-induced production of SARS-CoV-2 vital entry proteins, essentially ACE2, is still debatable[13]. A direct viral cytotoxic effect on hepatocytes appeared to induce mitochondrial and endoplasmic reticulum damage (swelling and distention accordingly), decreased glycogen granules and malfunction of the cell membrane in hepatocytes[31,33,34,42]. As in dysmetabolic conditions, the homeostasis of the liver and metabolic parameters are already impaired. This could have an additional effect on NAFLD. Finally, moderate microvesicular steatosis, along with other histopathological findings in deceased COVID-19 patients, may indicate additional pathways of metabolic derangement and liver damage[73].

In addition, the hyperinflammatory status in COVID-19 may act as a "second hit" to an inflamed fatty liver, causing "acute-on-chronic" steatohepatitis with increased aminotransferases[13,74]. Immune-mediated liver injury is caused by proinflammatory cytokines, chemokines and inflammatory cells produced against the SARS-CoV-2, which are also increased in obesity (IL-6, tumour necrosis factor)[32,34,75]. This may explain the finding that the C-reactive protein to albumin ratio was an independent risk factor for mortality in NAFLD patients with COVID-19 in one study[6]. Another significant immunological mediator of damage is viral-induced T cells (CD8)[31,32].

Another major cause of liver injury in COVID-19 appears to be coagulation disruption and endothelial damage[31,73]. Obesity is associated with hypercoagulation/thrombotic status dysregu-

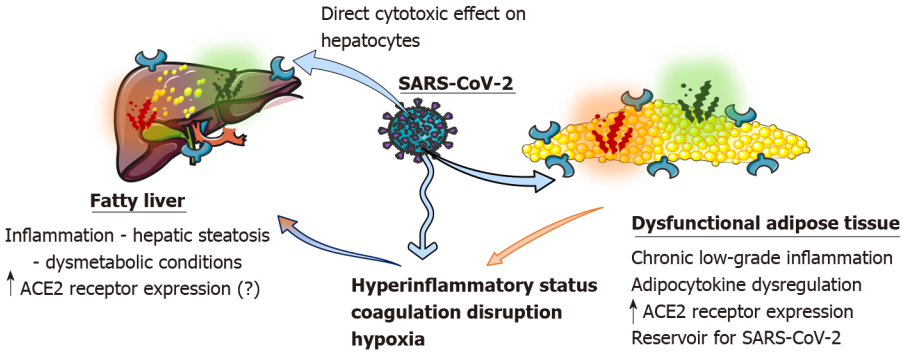


Figure 1 Potential mediators of liver damage associated to coronavirus disease 2019, fatty liver, and obesity. ACE2: Angiotensin converting enzyme 2; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2. Parts of the figure were drawn by using pictures from Servier Medical Art (smart.servier.com).

lation due to increased circulating concentrations of factor VII, von Willebrand factor and fibrinogen [13]. Ischemia-induced hypoxic liver injury most likely promotes the establishment of microvascular thrombosis[13,63]. Hypoxia caused by pneumonia is another major cause of liver damage[31]. This could be made worse in the case of COVID-19 and obesity, which is linked to obstructive sleep apnea and lower oxygen levels[28].

Ultimately, other factors may contribute to liver damage. It could be the end result of antivirals, anti-inflammatory medications, anticoagulants, antibiotics and other treatments used to treat underlying cardiometabolic chronic conditions and utilized during the COVID-19 infection, leading to liver injury [31]. Finally, dysbiosis and gut microbiota changes that usually occur in obesity could be exacerbated during COVID-19 and due to various medications[31,76]. A summary of the mechanisms can be found in Figure 1.

FUTURE PERSPECTIVES

Future studies should focus on elucidating and comparing a variety of populations with various body mass indexes (including normal weight, overweight and various degrees of obesity) because to date few studies have examined the presence of liver steatosis in patients with overweight or obesity[77]. Furthermore, the extent of underlying liver damage (NASH, liver fibrosis, or even liver cancer) should be determined in people who have pre-existing NAFLD and to monitor the severity of the COVID-19 infection outcomes. Finally, it would be of great interest to examine the potential differential impact of COVID-19 infection due to different viral variants on individuals with NAFLD and other liver manifestations, as these studies are sparse and focus on cirrhosis of various aetiologies[78].

CONCLUSION

In conclusion, the poor clinical outcomes of COVID-19 and the greater risk of pathological LFTs seen in people with obesity and/or NAFLD are likely caused by immune activation and a tendency toward a proinflammatory state during obesity as well as by metabolic and immune-mediated parameters that place the liver at risk. Thus, obesity should be prevented, especially in the context of the COVID-19 pandemic.

FOOTNOTES

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ORCID number: Eleni Karlafti 0000-0001-7094-0338; Petros Papalexis 0000-0003-3565-4066; George Fotakopoulos 0000-0002-2491-2886; Vasileios Lekakis 0000-0002-6105-4147; Stavros P Papadakos 0000-0003-1583-1125; Evangelos Cholongitas 0000-0002-3645-582X; Vasiliki E Georgakopoulou 0000-0003-0772-811X.

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OPINION REVIEW

Medical consultation in ulcerative colitis: Key elements for improvement

Yago González-Lama, Elena Ricart, Ana Cábez, Pilar Fortes, Susana Gómez, Francesc Casellas

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Yago González-Lama, Inflammatory Bowel Disease Unit, Department of Gastroenterology and Hepatology, Hospital Universitario Puerta de Hierro-Majadahonda, Madrid 28222, Spain

Elena Ricart, Inflammatory Bowel Disease Unit, Department of Gastroenterology, Hospital Clínic, Barcelona 08036, Spain

Elena Ricart, Francesc Casellas, Biomedical Research Networking Center in Hepatic and Digestive Diseases (CIBERehd), Barcelona 08036, Spain

Ana Cábez, Pilar Fortes, Department of Medical, Pfizer Spain, Madrid 28108, Spain

Susana Gómez, Servicio de Reumatología, Hospital Universitario de Salamanca, Salamanca 37007, Spain

Francesc Casellas, Department of Gastroenterology, Vall d'Hebron Research Institute, Barcelona 08035, Spain

Corresponding author: Francesc Casellas, MD, Doctor, Department of Gastroenterology, Vall d'Hebron Research Institute, Pg Vall d'Hebron 119-129, Barcelona 08035, Spain.
fcasellas@vhebron.net

Abstract

Ulcerative colitis (UC) is a chronic inflammatory disease with a high impact. In order to improve patient outcomes, the clinician-patient relationship in daily practice is critical. Clinical guidelines provide a framework for UC diagnosis and treatment. However, standard procedures and the medical content focused upon medical consultations in UC patients has not yet been defined. Moreover, UC is a complex disease, given that patient characteristics and patient needs have been proven to vary during clinical consultation since establishing the diagnosis and upon the course of the disease. In this article, we have discussed the key elements and specific objectives to consider in medical consultation, such as diagnosis, first visits, follow-up visits, active disease patients, patients on topical therapies, new treatment initiation, refractory patients, extra-intestinal manifestations, as well as challenging situations. The key elements have been mentioned to comprise effective communication techniques, motivational interviewing (MI), as well as information and educational aspects, or organizational issues. The key elements to be implemented in daily practice were reported to comprise several general principles like duly prepared consultations, in addition to honesty and empathy with patients, as well as effective communication techniques, MI, information and

educational points, or organizational issues. The role of other healthcare professionals such as specialized nurses, psychologists, or the use of checklists was also discussed and commented on.

Key Words: Ulcerative colitis; Patient experience; Shared decision making; Medical consultation; Motivational interviewing; Patient education

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Core Tip: Nowadays, patient-centered medical care has become accepted as a preferred care model, especially concerning chronic and heterogeneous diseases like ulcerative colitis. Clearly, medical consultations can substantially be improved by defining objectives along with the patients and adapting decisions and actions to this specific context. This comprises disease clinical features and stage, such as diagnosis, flare, and others, disease impact and patient's needs, as well as patient's opinions and preferences. Moreover, improvement can be obtained if we put into practice different resources that have demonstrated their efficacy in improving patient care such as effective communication techniques, motivational interviewing, or the use of checklists.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory disease that affects approximately 250 people out of 100000[1,2]. Different studies have reported the great impact of UC on patients, health systems, and society[3-6].

Currently, patient-centered medical care is synonymous with good-quality care, and it has become accepted as a preferred care model[7]. Effective clinician-patient communication is one of the cornerstones of patient-centered care, with proven impact on treatment adherence[8] and patient outcomes[9]. Key factors of effective communication have been shown to include patient understanding, trust, and respecting patients' preferences, in addition to shared decision-making. These factors are instrumental in influencing health outcomes like increased treatment adherence or better self-care skills[10].

On the other hand, UC characteristics and needs have been shown to greatly vary since the time the diagnosis has been made and during the disease course. Indeed, a recent publication demonstrated that patient preferences with respect to the disease management, disease information, as well as other items, differs depending on the context[11]. As a consequence, clinician-patient communication and decisions should similarly be adapted in daily practice.

Taking into account all of this information, we sought to define particular clinical scenarios and key elements to be considered upon medical consultation. For this purpose, we conducted a literature review and gathered expert's opinions. Although this work is focused on UC, most of the results can be applied to patients with other types of inflammatory bowel disease (IBD).

OVERARCHING PRINCIPLES IN A MEDICAL CONSULTATION FOR UC

Along with a set of overarching principles (Table 1), we have proposed eight particular clinical scenarios for which key elements and specific attitudes should be accounted for (Table 2).

Shared decision-making is increasingly advocated as an ideal model of therapeutic decision-making. In this model, the physician has the responsibility to inform the patients and provide them advice, whereas the actual decisions on how to act on this information are being made in a collaborative work environment between the patient and physician[12].

Among overarching principles during clinical consultation (Table 1), apart from an appropriate environment and preparation to the consultation, honesty and empathy with patients are essential, as is the adaptation of the physician's language and terms to the patients' understanding by avoiding technical terms that may not be fully understood. These features should always be considered as being dynamic when providing care to UC patients.

Taking into account UC's complexity, impact, and related treatments, in addition to considering non-adherence rates, it is essential to include behavioral interventions that likely improve patient outcomes

Table 1 Overarching principles for the clinical consultation in patients with ulcerative colitis

No.	Overarching principles
1	Appropriate environment where the clinical consultation takes place (tidy, clean, a chair for accompanying person, *etc.*)
2	Motivational interviewing
3	Use of appropriate verbal and non-verbal language
4	Good manners (salutation, face to face interaction, *etc.*) and empathy
5	Be honest about what we know, what we do not know and describe actions regarding what to do/what to avoid
6	Ideally prepare and organize the clinical consultation before seeing the patient (medical history, check labs, imaging tests, *etc.*)
7	Establish the objectives of the clinical consultation:
	Adapted to each clinical scenario
	Adapted (including the message) to each patient's features
	Taking into account patient's opinion and preferences

[8,13,14]. One such intervention is motivational interviewing (MI)[15]. MI is a directive patient-centered style of counselling, designed to help people further explore and resolve ambivalence about behavior changes. MI involves providers communicating in supportive, caring, and empathic ways to resolve a patient's ambivalence in view of health behavior change[16]. Specifically, providers ask, listen, and inform their patients when attempting to elicit motivation towards changes[16]. A systematic literature review involving patients with IBD has suggested that MI can be effective in improving outcomes for individuals suffering from IBD, given that patients displayed improved adherence rates and greater advice-seeking behavior, perceiving providers as having more empathy and better communication skills [15].

CLINICAL SCENARIOS AND KEY ELEMENTS DURING CLINICAL CONSULTATION FOR THE DIAGNOSIS/FIRST VISITS

The first scenario pertains to diagnosis/first visit(s) (Table 2). The emotional impact that is conveyed to any patient and environment when being confronted with the UC diagnosis is usually huge[3,17]. In addition to the initial anxiety and uncertainty, numerous patients report experiencing depressive symptoms and mental blocks after having become aware of their diagnosis[17,18]. Therefore, it is crucial to calm down the patients and help them cope with their new situation[19]. Given this context, effective clinician-patient communication has been shown to overcome barriers, while assisting patients in the decision-making about their care[9]. Communication between clinicians and patients is an interactive process upon which clinicians should listen, be open, receptive, and non-judgmental with patients[9]. Effective communication will contribute to build confidence.

In this scenario, it is also crucial to provide a minimum of information about the disease, its extra-intestinal manifestations and treatments, which should be adapted to the patient characteristics/needs and conducted in a constructive way, while also collecting data about the patients' support, especially concerning their family. On the other hand, a reasonable selection of the information to be provided is similarly advisable, thereby giving the patient the opportunity to adequately assume each issue and learn more about it[8,11]. Similarly, the same should be done with respect to the therapeutic plan by sharing treatment decisions and providing positive messages, such as: "Currently, there are several treatment options for this disease, and our objective is to improve your situation". The concept of "chronicity" should also be introduced by using expressions like: "while this is a long-term process, we can control the disease activity and avoid disease progression and symptoms", *etc.* Regarding health education, general information about diet, hygiene, healthy lifestyle, and other features should be provided[20].

An IBD specialized nurse working in a dedicated unit is likely able to improve all the information that we provide to the patient, resolve fears and doubts, and ameliorate adherence to treatments, care, and healthy habits[21]. Often, patients leave the doctor's office with large amounts of information and they feel overloaded. Therefore, changing the environment and providing a nursing summary can be very useful[19,22]. For communication to be optimal, we should try to additionally give the information in writing, at least with respect to what is essential. Reliable sources on the Internet should also be offered. In this context, many patients seek information on the disease from the internet. However, many times this information is inaccurate and even false, which might produce a negative impact on patients[23].

Table 2 Adapting the consultation to the different clinical scenarios when managing patients with ulcerative colitis

No.	Clinical scenario	Objectives	Key elements
1	Diagnosis/first visits	To engage patients in the management of their disease (treatments, visits, labs, image techniques, contact data, *etc.*); To inform wisely patients; To collect relevant data about the disease, patients and their environment	Use open questions rather than direct questions; Practice empathy: a climate of trust will make the patient feel comfortable and more information will come out naturally; When informing patients go as far as the patient needs or wants, *e.g.*, "What do you know about UC?"; Explore people's understanding, reactions and opinions about UC information; Explain the therapeutic plan (treatment objectives and goals, available therapies including surgery, response evaluation, *etc.*); Prepare the consultation: check labs, imaging tests or other available data before seeing the patient and prepare specific attitude, procedures or messages
2	Follow-up visits	To evaluate response to treatment (induction/maintenance), safety and adherence; In the induction phase, to ensure that the patient has achieved the pre-defined objective/goal; In the maintenance phase, to assure that the patient is really in remission and adherent; To explain test results	Use open questions; Solution-focused approach; Let the patients express themselves about their life (family, partner, work/studies, social life, traveling, diet, *etc.*): *e.g.*, "How are you doing? Are you doing everything you would like to do?"; Be open to patients asking questions; Detail the therapeutic and follow-up plan (*e.g.*, colonoscopy for colon cancer surveillance as indicated); Assess sexuality and fertility issues; Assess pregnancy desire: *e.g.*, when informing about drugs; Practice empathy; Prepare the consultation (see point 1)
3	Patients with active disease	To assess carefully the impact of UC on the patient's life (family, partner, work/academic, social life, traveling, diet, *etc.*); To adapt the treatment and patient's environment	Explain the therapeutic plan in detail but mainly explain and agree on what the objectives are, treatment goals and options, including surgery (informed shared decision making); Practice empathy; Prepare the consultation (see point 1)
4	Patients on topical therapies	To look for treatment acceptance and adherence	Explain in detail and clearly that topical therapies can be very effective with few adverse events (very good risk/benefit balance); Solution-focused approach; Discuss about the costs, explain their mechanism of action and options to improve comfort; Evaluate the patient's opinion and preferences; Practice empathy
5	Start of a new treatment	To assess carefully the impact of UC on the patient's life (family, partner, work/studies, social life, traveling, diet, *etc.*); To adapt the treatment and patient's environment; To fully understand the implications of starting a new treatment (changes in daily life, *etc.*)	Explain in detail and clearly the therapeutic plan but especially safety issues and new adaptations in patient's life; Solution-focused approach; Practice empathy; Prepare the consultation (see point 1)
6	Refractory patients	To assess carefully the impact of UC on the patient's life (family, partner, work/studies, social life, traveling, diet, *etc.*); To adapt the treatment and patient's environment	Explain the therapeutic plan in detail but mainly explain and agree on how far we are going to go, which are the treatment goals and options, including surgery (shared decision making); Solution-focused approach; Be especially cautious as non-standard treatments/strategies might be considered (*e.g.*, off-label uses, clinical trials, *etc.*); Spread positivity, make the patients feel accompanied; Screen for depression/dysthymia; Practice empathy; Prepare the consultation (see point 1)
7	Extra-intestinal manifestations	To inform patients about other symptoms or diseases	Explain the therapeutic plan in detail but mainly explain and agree on how far we are going to go, which are the treatment goals and options, including surgery (shared decision making); Solution-focused approach; Introduce the concept of multidisciplinary and coordinated care; Practice empathy; Prepare the consultation (see point 1)
8	Patients with negative feelings or mistrust	To win the patient over; To be patient but solid	Consider referring the patient to a psychologist; Explore fears, insecurities, frustrations; Do not confront the patient; Practice empathy; Explain the therapeutic plan in detail but mainly explain and agree on how far we are going to go, which are the treatment goals and options, including surgery (shared decision making)

For all sections, we start from the fundamentals of shared decision-making. UC: Ulcerative colitis.

There are several phases and common emotional reactions to the diagnosis, including denial, frustration, anger, and guilt; it is therefore essential to be attentive to the patient's emotional response [24]. In addition, it must be kept in mind that the patient faces significant losses, such as loss of health, of previous lifestyle, of quality of life, *etc.*

At the first visit, the patient should be offered the possibility to access the consultation, telephone, nurse, e-mail, or walk-in visits, as this has been shown to improve the patient's confidence in the care team[25].

Finally, physicians should keep in mind patient's opinions and preferences[11]. Therefore, it could be very useful to explore their beliefs, needs, and expectations. Moreover, it is advisable to leave some time for the patients, enabling them to ask questions during the consultation and encouraging them to "make a list of questions" if necessary for the next consultations[11].

CLINICAL SCENARIOS AND KEY ELEMENTS DURING CLINICAL CONSULTATION IN FOLLOW-UP VISITS

Physicians should continue addressing UC's impact on patients during the entire course of the disease. Indeed, during follow-up visits, patients may already be more aware of the disease's impact; they may thus be more comfortable to express it[22]. Apart from negative emotions, other problems may arise, such as those related to sexuality, sleep, work, or social life. In this context, it is vital to address, in male and female patient's sexuality issues and conception wishes, in order to optimize pre-conceptional, pregnancy and post pregnancy counselling, including the monitoring and therapeutic management of these patients[26]. Assessment of emotional aspects on the other hand should be an integral part of follow-up visits.

The objectives of pharmacological UC treatments are usually focused on controlling or at least reducing physical symptoms. Nevertheless, like in any other type of chronic disease, other psychological and social factors may influence the course of the disease[27]; they thus deserve appropriate attention. A central issue at this point is the assessment of treatment response upon induction and maintenance, in addition to evaluating safety and treatment adherence. While it is crucial to ensure that the patient has achieved the treatment goal, the treatment's impact on safety must likewise be assessed. In addition, considering the high rate of poor adherence in UC[28], health professionals should clearly address this issue, while actively involving the patients in implementing effective and individualized interventions to improve adherence[8].

Similarly to the first visits, information and education are vital in daily consultations, which should be appropriately adapted to the patients' characteristics and needs[11]. These consultations could be an appropriate opportunity to explain the test results to patients, as well as the role of colonoscopy for colon cancer surveillance.

CLINICAL SCENARIOS AND KEY ELEMENTS DURING CLINICAL CONSULTATION IN PATIENTS WITH ACTIVE DISEASE

Relapses are common in UC, and they are associated with a significant impact on different aspects of patients' lives[3,18]. Thus, the impact of disease activity should be carefully assessed, and physicians should thus perform therapeutic adaptations that should be discussed with the patients. Accordingly, the patients will thus feel accompanied.

The emotional impact should be included in the information/education package as disease symptoms. Physical remission does not imply the absence of psychological comorbidity in a high percentage of patients[29]. Therefore, it is essential to provide information about physical symptoms, including diarrhea, abdominal pain, and fever; emotional symptoms like anxiety, depression, irritability, mood changes, and behavioral symptoms, such as decreased activity, restriction of social relations, and isolation[19].

On the other hand, psychological treatments exert beneficial therapeutic effects, as they decrease the worsening cycle of symptoms and positively influence the patients' general well-being[13].

The integration of psychosocial factors into routine clinical practice is likely instrumental in improving quality of life of IBD patients[19].

Considering the treatment, informed shared decision-making is especially relevant in this domain [30]. At this point, physicians may be confronted with patients who are refractory to different treatment lines or types. Apart from detailed information about different treatment options, including surgery, it is even more relevant to discuss the treatment goals and get approval about them ("What is the target we want to achieve?").

CLINICAL SCENARIOS AND KEY ELEMENTS DURING CLINICAL CONSULTATION IN PATIENTS ON TOPICAL THERAPIES

The efficacy of topical therapies, especially in patients with moderate UC, has been extensively described[31]. However, the adherence to topical therapies appears to be lower when compared with oral treatments[32]. This might be related to reluctances, taboos, or other factors. Asking patients about their feelings regarding topical therapy may help anticipate a lack of adherence, thus providing the physician with the opportunity to adapt topical therapy to each patient[8].

Discussing treatment goals with the patient and setting a temporal limit may be of help when addressing topical treatments[8].

CLINICAL SCENARIOS AND KEY ELEMENTS DURING CLINICAL CONSULTATION WHEN STARTING A NEW TREATMENT

During follow-up, a number of patients may require a change in therapy due to a lack or loss of efficacy, or safety reasons. This might be associated with changes in daily life, particularly when the first immunomodulator or biologic agent is being considered. Along with all that has been previously discussed regarding patients with active disease, it must be ensured that the patient has properly understood the implications of starting a new treatment.

When starting new treatments, false beliefs such as "if I change treatment, I will worsen" or "the disease is more serious" should be dismissed. Concerns about the limit of treatments to be used, possibility of surgery, or need for an ostomy should also be addressed.

CLINICAL SCENARIOS AND KEY ELEMENTS DURING CLINICAL CONSULTATION IN REFRACTORY DISEASE PATIENTS

In daily consultation, some patients may display several failures to treatment, including biologics; they may experience frustration and despair[22]. Therefore, it is crucial to distribute positivity but also reality, given that new treatments with different mechanisms of action are being made available[19]. In some centers, authorized treatments/strategies might be considered, including off-label uses, clinical trial participation, as well as other treatments. Screening for depression/dysthymia may be required given such a clinical scenario.

It is necessary to train non-psychologists in assessing and managing emotional aspects, such as identifying symptoms related to stress, anxiety, and depression, which are among the most common [19].

CLINICAL SCENARIOS AND KEY ELEMENTS DURING CLINICAL CONSULTATION IN PATIENTS WITH EXTRA-INTESTINAL MANIFESTATIONS

As estimated, up to 50% of IBD patients experience at least one extra-intestinal manifestation, which can even occur before the IBD is being diagnosed[33]. In view of an early detection, patients should be aware that different symptoms or signs may occur, which are actually related to UC, while deserving specific actions, including consultation with other specialists. In addition, information about multidisciplinary care and coordination among health professionals will likely help control fears and doubts[19,25].

CLINICAL SCENARIOS AND KEY ELEMENTS DURING CLINICAL CONSULTATION IN PATIENTS WITH NEGATIVE FEELINGS OR MISTRUST

Finally, some patients may experience negative emotions, as they are reluctant to accept existing problems or proposed solutions, disadvantages, insufficient answers, *etc*. Such scenarios can be particularly frustrating during the consultation. Considering these patients, one should not try to "convince them", given that they are likely to become even more isolated. Instead, making them understand that we are on their team, and that they are actually responsible for their disease management is crucial[25]. In some cases, a referral to a psychologist could be an appropriate solution[34].

Healthy lifestyle information should be based on guidelines on how to handle stressful situations. Illness is a source of stress, and stress is considered a risk factor for increased severity of physical symptoms[13]. For this purpose, it is advisable to refer to reliable web pages, brochures, conferences, workshops, or even patient associations, as some of them do provide psychological assistance.

CONCLUSION

UC is a chronic, heterogeneous, and evolving disease, whose clinical features and disease impact, as well as patients' needs, opinions, and preferences may completely differ depending on the disease status[3-6,11]. In daily practice, the clinical picture of patients at early disease stages compared with that of patients with long-standing disease is not at all the same. Therefore, the physicians' attitudes and decisions should take these facts into account during the clinical consultation. Moreover, there is growing evidence suggesting that there is improved performance in this area to the benefit of both patients and clinicians[9,35].

Effective communication has emerged as a very valuable tool to superior informed decisions by patients during the clinical consultation[9]. Communication is crucial in establishing trust with UC patients, gathering patient information, addressing patient emotions, and assisting patients in the decision-making about their care[9,35]. This article sought to discuss some guidelines for physicians about how to implement effective communication during clinical consultation, as adapted to several clinical scenarios in which the objectives, attitudes, and messages most likely differ. Simple, practical, and implementable key elements including empathy, open questions, patient-centered care and information, solution-focused approach, or MI have herein been proposed.

Along with effective communication, we also suggest other actions like preparing the consultation or using appropriate verbal and non-verbal language, which have already demonstrated an effect on patient's management[36].

Some of the strengths of this work are as follows: innovation in the treatment of UC patients, how to initiate an updated patient management model, using a check list to prepare the different consultations, as well as having a specialist nurse and psychologist as external evaluators. The article's weaknesses comprise the lack of clinical evaluation or the absence of other scenarios that would have been interesting to address, such as the transition of the child to adult unit or continuity of care during admissions. Nevertheless, we consider that these items were beyond the paper's scope of interest.

In conclusion, research in UC clearly shows that it is essential to build a physician-patient partnership, focusing on the problem at hand, laying out the treatment options available along with their benefit/risk balance, eliciting the patient's views and preferences on treatment options, and agreeing on a course of action *via* shared decision-making. For these purposes, specific communication skills are necessary; they all can be learned and should be implemented during clinical consultations. We are confident that the guide provided in this article for clinical consultation will help physicians better manage UC patients.

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Country/Territory of origin: Spain

ORCID number: Yago Gonzalez Lama 0000-0003-3896-7914; Elena Ricart 0000-0003-3354-1594; Ana Cábez 0000-0003-0808-8855; Pilar Fortes 0000-0001-9420-321X; Susana Gómez 0000-0002-5337-2970; Francesc Casellas 0000-0003-3196-039X.

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REVIEW

Resistance to targeted therapy in metastatic colorectal cancer: Current status and new developments

Yuan-Ling Tang, Dan-Dan Li, Jia-Yu Duan, Lei-Ming Sheng, Xin Wang

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Yuan-Ling Tang, Dan-Dan Li, Jia-Yu Duan, Lei-Ming Sheng, Xin Wang, Department of Radiation Oncology, Cancer Center, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China

Yuan-Ling Tang, Dan-Dan Li, Jia-Yu Duan, Lei-Ming Sheng, Xin Wang, Department of Abdominal Cancer, Cancer Center, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China

Corresponding author: Xin Wang, MD, PhD, Doctor, Department of Radiation Oncology, Cancer Center, West China Hospital of Sichuan University, No. 37 Guoxue Lane, Wuhou District, Chengdu 610041, Sichuan Province, China. 2079248480@qq.com

Abstract

Colorectal cancer (CRC) is one of the most lethal and common malignancies in the world. Chemotherapy has been the conventional treatment for metastatic CRC (mCRC) patients. However, the effects of chemotherapy have been unsatisfactory. With the advent of targeted therapy, the survival of patients with CRC have been prolonged. Over the past 20 years, targeted therapy for CRC has achieved substantial progress. However, targeted therapy has the same challenge of drug resistance as chemotherapy. Consequently, exploring the resistance mechanism and finding strategies to address the resistance to targeted therapy, along with searching for novel effective regimens, is a constant challenge in the mCRC treatment, and it is also a hot research topic. In this review, we focus on the current status on resistance to existing targeted therapies in mCRC and discuss future developments.

Key Words: Colorectal cancer; Targeted treatment; Resistance; New development

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Core Tip: Colorectal cancer (CRC) is one of the most lethal and common malignancies in the world. Chemotherapy has long been the mainstay of CRC treatment. However, since chemotherapy is not a specific regimen, it will produce systemic toxicity. Following, with the advent of targeted therapy, the prognosis of CRC has been improved significantly. Although targeted therapy also develop drug resistance, more and more novel targets and combination regimens are being explored over the past 20 years. In this review, we summarized resistance to exiting targeted therapy, and discussed future developments in CRC.

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INTRODUCTION

Colorectal cancer (CRC) is the third most prevalent malignancy and the second most lethal cancer worldwide[1]. Surgery is the primary treatment option and has the potential to be curative, especially for patients in the early stage of the disease[2]. However, most patients suffer from advanced/metastatic tumors, with a five-year overall survival (OS) rate of approximately 13%[3]. Systemic chemotherapy is the cornerstone of treatment for these patients, resulting in a median OS (mOS) of 17-23 mo[4-6]. The prognosis of patients with mCRC has improved with the addition of targeted therapies, such as antibodies against epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF), and tyrosine kinase inhibitors (TKIs)[7,8].

CRC is a heterogeneous disease with multiple molecular features, requiring individual targeted approach for achieving effective disease control and good survival rates[9]. This review presents the standard targeted therapy options and corresponding resistance mechanisms, and discuss the promising targeted agents for mCRC.

CURRENT TREATMENT FOR COLORECTAL CANCER

Conventional treatments for CRC include 5-fluorouracil (5-FU)-based chemotherapy including leucovorin, 5-FU, and oxaliplatin (FOLFOX), leucovorin, 5-FU, and irinotecan (FOLFIRI), and capecitabine and oxaliplatin (CAPOX)[10,11]. CRC chemotherapy has certain limitations due to systemic toxicity, low population-specific selectivity, and unsatisfactory response rate. Targeted therapies, including small molecule inhibitors and monoclonal antibodies (mAbs), are effective approaches following chemotherapy. The antiangiogenic drugs bevacizumab and anti-EGFR regimens have been successively approved by the Food and Drug Administration (FDA) for both first- and second-line CRC treatment[12-15]. In the Biomarkers of Nutrition for Development (BOND) trial, cetuximab showed clinically significant activity to improve the response rate and progression-free survival (PFS) in patients with irinotecan-refractory CRC; the most frequently observed adverse events were diarrhea, asthenia, and acne-like rash[12]. Panitumumab has a lower risk of hypersensitivity reactions than cetuximab, because panitumumab is a fully humanized antibody unlike others, which are murine-human chimeric antibodies[16]. The efficacy and safety of panitumumab plus fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) has been proven in the PRIME trial[15,17]. Randomized controlled trials have shown that bevacizumab with chemotherapy could significantly increase OS and PFS in patients with CRC[14,18,19], and both patients with kirsten rat sarcoma 2 viral oncogene homolog (KRAS) mutations and those with the wild-type (WT) genotype could benefit from bevacizumab[20,21]. Besides, bevacizumab is the only antiangiogenic drug approved for the first-line treatment of CRC; some other novel anti-VEGF drugs have shown favorable results in second-line treatment of CRC, such as aflibercept and ramucirumab[22-24]. The main toxic effects of bevacizumab are cardiotoxicity, including hypertension and bradycardia. In addition, TKIs, including regorafenib and fruquintinib, were also options for CRC patients, which have been approved for third-line treatment[25,26]. TAS-102 is a novel oral drug that is a combination of trifluridine and tipiracil. Trifluridine is a cytotoxic nucleic acid analog that leads to DNA dysfunction, whereas tipiracil is a thymidine phosphorylase that inhibits enzyme degradation.

RESISTANCE TO TARGETED THERAPY IN CRC

EGFR pathway

EGFR is a member of ERBB (erythroblastosis oncogene B)/HER (human EGFR) family. Overexpression of EGFR has been detected in 25%-77% of CRCs[27]. Notably, subgroup analysis from the CRYSTAL and PRIME trials revealed that anti-EGFR therapy only benefited the population with WT RAS mutation[13,15]. Patients with BRAF mutations failed to respond to anti-EGFR therapy and BRAF mutations were independent of RAS mutations. Therefore, the European Society of Medical Oncology (ESMO) and National Comprehensive Cancer Network (NCCN) guidelines recommend anti-EGFR therapy only for patients with BRAF-WT and RAS-WT. Interestingly, left-sided CRC expressed more EGFR than right-sided CRC. The American Society of Clinical Oncology recommends that populations with left-sided tumors *vs* right-sided tumors seem to benefit more from anti-EGFR therapy and are associated with better prognosis[28]. It can be seen that not all patients are suitable for anti-EGFR therapy, and even though patients respond to it, resistance develops in 3-12 mo[29,30]. Gene mutations downstream of the EGFR signaling pathways are the main causes of resistance to anti-EGFR therapy, including RAS/RAF/MEK and PI3K/AKT/mTOR[31-33]. In addition, the activation of compensatory pathways of EGFR, such as ERBB2 and MET, is also the reason for the drug resistance to anti-EGFR[34-36]. In addition to the intrinsic mechanisms mentioned above, microenvironmental plasticity also conferred cetuximab and panitumumab resistance[37].

VEGF pathway

Angiogenesis is essential for tumor initiation, development, and metastasis. Overexpression of the VEGF ligand family and their binding to tyrosine kinase receptors (VEGFR-1, VEGFR-2, and VEGFR-3) result in endothelial cell growth and migration[38]. The activation of alternative signaling pathways and production of angiogenesis-related proteins may cause resistance to antiangiogenic therapy. Investigations demonstrated that both patients with KRAS-mutated-type (MT) and those with KRAS-WT could benefit from bevacizumab, which was different from cetuximab[20,21,39]. Placental growth factor is a pivotal indicator of anti-VEGF resistance, which is part of the reason that aflibercept is more effective than bevacizumab in xenograft models[40,41]. In addition, abnormal increases in angiopoietin-2 have been observed in many types of tumors, including CRC, and have been shown to be associated with resistance to bevacizumab[42-44]. Activation of the fibroblast growth factor-1 (FGF)/FGFR pathway has the ability to promote cell survival and migration in both normal and malignant tissues, which is also seen in anti-VEGF resistant population[45,46]. The mechanism most closely associated with the loss of anti-VEGF effectiveness is compensatory stimulation of the c-MET pathway, in which case, single-agent c-MET suppression might be beneficial[47].

NEW DEVELOPMENTS IN COLORECTAL CANCER

Overcoming anti-EGFR therapy resistance

In general, strategies to overcome resistance to anti-EGFR regimens include the following: Novel EGFR-targeted drugs, multi-targeted regimen combinations, metabolic regulators, and immunotherapy.

EGFR ligands and EGFR: The expression of EGFR ligands potentially correlates with efficacy of anti-EGFR therapy[48,49]. Thus, the development of new mAbs that can target different EGFR epitopes promises to reverse anti-EGFR resistance. Among different EGFR somatic sequence changes located in the extracellular domain (ECD), S492R affects cetuximab binding, but does not interrupt panitumumab binding, suggesting a rechallenge with panitumumab in patients resistant to cetuximab and developing an S492R mutation[50]. MM-151, an mAb that engages multiple epitopes on EGFR, interferes with EGFR signaling and suppresses tumor activity in a preclinical study[51]. Sym004 is a combination of two distinct anti-EGFR mAbs and showed significant superiority in suppressing phosphorylation of EGFR ligand and inhibiting EGFR downstream pathways in a tumor xenograft model[52,53]. A multicenter, phase II clinical trial explored the efficacy of Sym004 in patients with mCRC with secondary resistance to anti-EGFR therapy. Although Sym004 did not improve prognosis compared with the control group (capecitabine, fluorouracil, and best supportive care), subgroup analysis demonstrated that patients without EGFR ECD mutations had benefited in OS with Sym004 therapy[54]. Necitumumab is another approved EGFR antibody that can integrate with S468R, the most common cetuximab resistance substitution[55]. Necitumumab plus mFOLFOX6 showed favorable survival with manageable toxicity in the first-line treatment of mCRC patients[56].

Gene mutations in downstream pathways

Targeting RAS mutant CRC: RAS mutations present in nearly half of CRC patients usually occur in KRAS, NRAS, and HRAS[33,57]. KRAS is the most frequent mutation (40%), predominantly in exon 2, codons 12 and 13, and less commonly in exons 3 and 4[57-59]. Although RAS mutations are negative predictive markers of anti-EGFR treatment activity and are associated with poor survival outcomes,

data have demonstrated that not all KRAS-mutated patients develop resistance to anti-EGFR treatment [60]. It is unclear whether patients with $KRAS^{G13D}$ gene mutations develop drug resistance[61-63].

RAS direct inhibitors: RAS proteins are small guanosine triphosphate (GTP) phosphatases (GTPases) and are turned on when GTP-bound[64]. In the activated state, RAS alters its conformation and activates downstream pathways to promote cell proliferation, migration, and survival. This is precise because of the high affinity of RAS for GTP/GDP and the lack of suitable small molecules targeting known allosteric sites[65]. Among the most common RAS mutations, $KRAS^{G12C}$ possesses a unique near-WT intrinsic GTPase activity and has recently been demonstrated to be druggable[66,67]. Sotorasib (AMG 510) is the first drug tested in clinical trials, which specifically and irreversibly inhibits $KRAS^{G12C}$. The anticancer activity of sotorasib in patients harboring the $KRAS^{G12C}$ mutation has been demonstrated in a phase I trial. In the subgroup with colorectal cancer, 3 of 42 (7.1%) patients achieved partial response (PR), 28 (66.7%) experienced stable disease (SD), and the mPFS was 4.0 mo[67,68]. Adagrasib (MRTX849) is another covalent $KRAS^{G12C}$ inhibitor that achieves an overall response rate (ORR) and disease control rate (DCR) of 17% (3/18) and 94% (17/18), respectively, in patients with $KRAS^{G12C}$ CRC [69]. In addition, a phase III trial assessed the effect of adagrasib plus cetuximab as second-line treatment for $KRAS^{G12C}$ MT mCRC[70]. Regrettably, $KRAS^{G12C}$ is found in only 4% of CRCs, and $KRAS^{G12D}$ is the most common type of CRC; thus, developing a specific effective inhibitor is of great significance in clinical practice[71]. The development of inhibitors directly targeting $KRAS^{G12D}$ is much more difficult than that targeting $KRAS^{G12C}$ because $KRAS^{G12D}$ lacks cysteine, which can covalently bind to small molecular drugs. MRTX1133 is the first noncovalent and specific inhibitor of $KRAS^{G12D}$ with preclinical data. The binding activity and selectivity of MRTX1133 were improved by optimizing the positions 2, 4, and 7 of pyrido[4,3-d] pyrimidine based on adagrasib. In the mouse models of xenograft tumors, MRTX1133 has been shown to inhibit KRAS signaling and exhibit robust antitumor activity[72]. In addition, Revolution Medicines have developed a covalent $KRAS^{G12D}$ inhibitor that targets the activated state of KRAS. RM-036 forms a tri-complex with chaperone cyclophilin A and the on-state of $KRAS^{G12D}$ and can selectively bind to aspartate of $KRAS^{G12D}$ mutants, effectively inhibiting signal transduction. Treatment with RM-036 resulted in dramatic tumor regression and a complete response in several $KRAS^{G12D}$ mutant xenograft models[73]. Overall, targeted therapies for $KRAS^{G12D}$ are in the early stages of research, and more clinical trials are needed. Recently, a pan-KRAS probe compound, BI-panKRAS3, demonstrated antitumor efficacy in CRC models with $KRAS^{G12D}$ and $KRAS^{G13D}$ mutations [74].

Targeting the downstream pathways: MAPK pathway: Inhibition of MAPK effectors is another strategy targeting RAS-MT CRC[75]. Trametinib, binimetinib, and cobimetinib, which are MEK inhibitors (MEKi), prevent MEK phosphorylation of ERK1/2, thereby preventing dimerization and nuclear translocation[76]. However, these drugs alone did not show favorable results in this subset of population[77-79]. Several studies have focused on simultaneous use of inhibitors of MEK and upstream RTK, such as combination of MEKi with anti-EGFR drugs[80,81]. Results from both *in vitro* and *in vivo* studies suggest that the combination of cetuximab and trametinib can induce KRAS-MT cell death[82-85]. Moreover, activation of the MAPK pathway may dysregulate the cell cycle *via* the cyclin-dependent kinase (CDK) pathway. Palbociclib, a CDK4/6 inhibitor, showed limited antitumor activity when used alone (0% ORR and 33% DCR)[63]. However, the combination of binimetinib and palbociclib exhibited synergistic suppression of cell growth in *in vivo* trials[86]. The efficacy of palbociclib and another CDK4/6 inhibitor, trametinib, was evaluated in a phase Ib study (NCT02065063). In a case report, a CRC patient with KRAS-MT achieved PR for up to 10.8 mo with this combination regimen[87].

Immunotherapy combination: KRAS mutations might promote cancer immune escape mechanisms, and they are also relevant to programmed cell death protein 1 (PD-1) and decrease the expression of programmed death-ligand 1 (PD-L1) in CRC with microsatellite instability (MSI)[88]. In preclinical models, sotorasib plus checkpoint inhibitor therapy (immune checkpoint inhibitors, ICIs) increased T cell infiltration and activation, thereby forming a tumor microenvironment (TME) that was highly sensitive to immune-checkpoint inhibition[89]. Similar results were observed with the combination of adagrasib and anti-PD-L[90].

Targeting RAS through metabolic pathways: KRAS mutations can drive metabolic reprogramming by increasing glucose uptake and protein content and exhibit a Warburg effect phenotype[91]. Fatty acid synthase (FASN) regulates lipid synthesis and is often upregulated in KRAS-MT tumors. A phase I trial explored the pharmacodynamic effects of TVB-2640 (a FASN inhibitor) in patients with resectable tumors (NCT02980029)[92]. Interestingly, as the first-line treatment for type 2 diabetes mellitus, metformin may have a chemo-preventive role in CRC[93]. In preclinical studies, metformin showed a synergistic effect with oxaliplatin in the *in vitro* models of colon cancer[94]. However, the effect of metformin on the survival of patients receiving chemotherapy after resection is still inconclusive[95-98]. Additional evidence from prospective randomized controlled trials is needed. It has been reported that cultured CRC cells with KRAS mutations exhibited induced energy crisis and cell death when exposed to high doses of vitamin C (AA)[99]. The favorable safety and preliminary efficacy of AA plus mFOLFOX6/FOLFIRI (irinotecan, 5-fluoruracil, and leucovorin) in mCRC have been proven in a phase I study. Relevant phase II and III studies are ongoing[100].

Targeting BRAF mutant CRC: BRAF mutations occur in approximately 12% of patients with CRC, and

more than 95% of these mutations manifest as V600E substitution[101-104]. Patients with BRAF-MT CRC are more common in women and in people diagnosed at an old age, and the primary tumors mainly occur on the right side and in advanced stages, most of which are mucinous adenocarcinomas. Moreover, BRAF-MT CRC mainly develops peritoneal and lymph node metastasis[105-107]. In addition, more than 50% of BRAF V600E mutant CRCs have a high MSI (MSI-H) status[108,109]. CRCs harboring these features are frequently sporadic and exhibit extensive DNA methylation of CpG islands[110,111]. In contrast, MSI status in the absence of BRAF V600E mutation is related to Lynch syndrome[112]. The mOS of patients with BRAF-MT is approximately 11 mo, which is lower than that of patients with BRAF-WT[106,107]. Notably, non-V600E BRAF is defined as a clinically special subgroup with a good prognosis, which occurs in 2.2% of all patients with CRC[107]. Several cases have provided evidence that patients with a non-V600 BRAF mutation can respond to EGFR inhibitors[113,114].

The NCCN guidelines recommend combination chemotherapy as the foundation of therapy for patients with BRAF-mutated CRC[8]. The Triplet plus Bevacizumab (TRIBE) study compared the effectiveness and safety of FOLFIRI plus bevacizumab *vs* FOLFOXIRI plus bevacizumab in untreated patients with mCRC. Sixteen patients received FOLFOXIRI plus bevacizumab, and 12 patients received FOLFIRI plus bevacizumab, with a median OS of 19.0 mo and 10.7 mo respectively[115]. However, these promising survival data were not confirmed in the TRIBE phase III trial[116]. In addition, a meta-analysis indicated that FOLFOXIRI with bevacizumab failed to show superiority compared to doublet combination plus bevacizumab in patients with BRAF-mutant tumors[117]. Therefore, there is insufficient evidence to suggest that triplet combination regimens are better than doublet chemotherapy as first-line treatment for BRAF V600E-mutated CRC.

Monotherapy strategies: Vemurafenib is an oral inhibitor of BRAF V600 kinase, approved by the FDA for metastatic melanoma patients, with an effective rate of 77.1%[118,119]. Nevertheless, vemurafenib as a single agent, showed limited activity in BRAF-MT mCRC, with a response rate of 5%[120]. This result illustrates that BRAF activation in mCRC is more intricate and heterogeneous than that in melanoma. The activity of BRAF inhibitors may cause feedback signaling *via* reactivation of EGFR, whereas melanoma cells express low levels of EGFR[121,122].

Concurrent blockade of BRAF and EGFR: Several studies have shown that EGFR inhibitors combined with BRAF inhibitors could be an effective strategy[122,123]. The efficacy of vemurafenib combined with panitumumab in 15 patients with chemo-resistant BRAF-MT mCRC. The results of this trial included ten cases of tumor regression and two cases of stable disease lasting over 6 mo. Additional studies exploring dual blockade of BRAF and EGFR showed response rates of 10%-39%[124]. Interestingly, adding irinotecan to the two-drug regimen can further inhibit tumor growth. In a phase I trial (NCT01787500), patients with pretreated BRAF-MT mCRC achieved sustained disease control after receiving this triple combination therapy, and the mPFS was 7.7 mo[125]. The SWOG1406 phase III trial recruited 160 patients with BRAF-MT mCRC and randomly assigned them to two treatment groups: 54 patients received irinotecan, cetuximab, and vemurafenib, while the others received cetuximab and irinotecan. The results showed that the ORR and DCR increased by 17% and 65%, respectively, with the addition of vemurafenib[126]. Accordingly, the 2018 NCCN guidelines recommended that mCRC patients with BRAF-MT could choose three-drug combination regimens: Irinotecan, anti-EGFR therapy, and anti-BRAF therapy (vemurafenib)[127]. Recently, a study found that RNF43 mutations could help prioritize patients with mCRC BRAF-V600E, who are more likely to respond to the anti-EGFR/BRAF combination. Further research is needed to explore the incorporation of this biomarker along with BRAF and microsatellite stable (MSS)/MSI status in routine testing[128].

Concurrent blockade of BRAF and MEK: The combination of BRAF and MEK inhibitors can also produce potentially favorable antitumor activity by enhancing inhibition of the MAPK pathway[129,130]. In the phase III BEACON study, 665 BRAF V600E-MT mCRC patients were enrolled and randomly divided into three groups: Encorafenib, binimetinib, and cetuximab; encorafenib and cetuximab; and irinotecan plus FOLFIRI or cetuximab (control group). The triplet therapy significantly improved the OS, with an mOS of 9.0 mo. The objective response rates of the triplet therapy, doublet therapy, and control groups were 27%, 20%, and 2%, respectively[131,132]. Based on these results, the FDA approved doublet regimens as second-line treatment for BRAF-MT mCRC therapy, while in Japan, both doublet and triplet regimens are approved[133]. The ANCHOR study is an ongoing trial to estimate the efficacy of encorafenib, binimetinib, and cetuximab for BRAF-MT mCRC in previously untreated patients and is also the first prospective study using BRAF inhibitor regimens as the first-line therapy[134]. Dabrafenib is another selective BRAF inhibitor competing against ATP[135]. Ryan *et al*[64] tested dabrafenib plus trametinib in 43 patients with BRAF V600-mutanted CRC, of whom five (12%) achieved partial response, and 24 (56%) achieved stable disease[136].

PI3K/AKT activation and PI3K/AKT inhibitors: PIK3CA encodes the p110α catalytic subunit of phosphatidylinositol 3-kinase (PI3K), found in 10%-20% of CRC patients[31]. PI3KA mutation and abnormal AKT/mTOR activation lead to reversal of EGFR-blocking effects in CRC. PIK3CA mutations usually occur in exons 9 and 20, and exon 20 mutations are associated with worse prognosis in KRAS-WT mCRC patients treated with cetuximab, whereas exon 9 mutations did not affect survival[31,137]. Experimental data suggest that aspirin suppresses prostaglandin-endoperoxide synthase 2 and

downregulates PI3K signaling activity[138]. Low-dose aspirin had a significant interactive effect on survival among patients with PIK3CA-MT CRC but not among patients with PIK3CA-WT[139]. This observation requires a prospective evaluation. PTEN (phosphatase and tensin homolog) is a negative modifier of the PI3K/AKT pathway, detected in 20%-40% of mCRC cases, the loss of which results in tumor growth by activating PI3K/AKT[140]. Tumors without mutations in KRAS, NRAS, BRAF, and PIK3CA are called "quadruple negative" CRCs, responding best to anti-EGFR therapy[80].

Compensatory feedback loop signaling

HER-2 amplification/overexpression: Amplification of the HER2 gene, which induces excessive PI3KCA/AKT/mTOR signaling, occurred in 5%-7% of CRC patients, mostly in RAS wild-type tumors [36,141,142]. The incidence of HER2 mutations was not related to patient age, sex, tumor stage, or anatomical location[143]. However, genomic profiling studies have reported that HER2 mutations are more common in high tumor mutation burden or MSI-H tumors[144,145].

Early clinical studies evaluating HER2 inhibitors with chemotherapy were terminated because of poor accrual[146,147]. It has been speculated that the low frequency of HER2 expression in CRC restricts the application of HER2-targeted therapy. Bertotti *et al*[141] found that HER2 positive patients were sensitive to dual regimens of trastuzumab and lapatinib but not to either agent alone. HERACLESA, a multicenter phase II trial, evaluated the efficacy of a combination of trastuzumab and lapatinib in mCRC patients with KRAS-WT and HER2 overexpression refractory to standard treatment[148]. HER2 positivity was defined by immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH): Tumors with a score of IHC 3 + in more than half of the cells, or with an IHC 2 +:CEP17 ratio > 2 in more than half of the cells[149]. The study achieved an ORR of 30% and showed encouraging survival outcomes: An mPFS of 5.0 mo and an mOS of 11.5 mo[148]. Based on these results, trastuzumab and lapatinib regimens were included in the 2019 NCCN Guidelines for CRC, and more clinical research has been triggered to optimize anti-HER2 regimens in this setting[8]. In the HERACLES-B study, a single-arm phase II trial, patients were treated with pertuzumab and trastuzumab (T-DM1). A total of 31 patients were evaluable, of whom 48% had more than four lines of previous therapies. Although the study did not meet its primary endpoint, with an ORR of 9.7%[150], it demonstrated a similar mPFS to other anti-HER2 regimens with low toxicity and high disease control. In the phase II MyPathway trial, patients also received pertuzumab and trastuzumab, and the ORR was only 8% in the KRAS-MT population compared to 40% in the KRAS-WT population, suggesting that patients with KRAS-WT mCRC benefited more from the combination of pertuzumab and trastuzumab than those with KRAS-MT mCRC[151,152]. The efficacy of pertuzumab plus trastuzumab was confirmed in the TRIUMPH trial. This was the first study to define HER2-positive mCRC based on tissue or circulating tumor DNA (ctDNA) analysis and to confirm the feasibility of using ctDNA to identify HER2 expression for therapeutic targeting[153].

Trastuzumab deruxtecan (T-DXd) is an antibody-drug conjugate formed by humanized anti-HER2 antibody with a topoisomerase I inhibitor payload. Its effectiveness was demonstrated in the DESTINY-CRC01 phase II trial[154]. Phase II DESTINY-CRC02 was designed to assess the efficacy of T-DXd in patients with HER2-amplified mCRC at a lower dose (5.4 mg/kg) compared to that in other studies. Preliminary data showed that T-DXd may be effective in RAS-MT mCRC, which is different from other trials of anti-HER2 regimens[155].

Tucatinib, a selective oral small-molecule inhibitor of HER2, has shown promising efficacy in HER2 positive metastatic breast cancer[156]. In xenograft models of patients with HER positive CRC, tucatinib showed significant antitumor activity. When combined with trastuzumab, the effect of tumor growth inhibition becomes more pronounced[156]. This provided the foundation for the MOUNTAINEER study, in which 26 patients with RAS-WT HER2 positive mCRC received tucatinib with trastuzumab. The results showed an ORR of 55%, with an impressive mPFS of 6.2 mo and OS of 17.3 mo, respectively [157,158].

MET amplification/activation and MET inhibitors: The phosphorylation of MET results in the activation of PI3K/AKT and RAS/RAF/MAPK to improve tumor cell growth[159]. The dual blockade of MET and EGFR could provide a new therapeutic strategy for CRC patients with acquired MET-driven resistance to anti-EGFR. A phase II clinical study evaluated the efficacy of tivantinib and cetuximab in 41 patients with secondary resistance to anti-EGFR and MET overexpression. In the first stage, the study showed promising results with a DCR of 52.4%. However, the study did not reach the primary endpoint during the second stage, and only four patients achieved an objective response instead of five as expected. Whereas, the survival results were still encouraging, with an mPFS of 2.6 mo and mOS of 9.2 mo, respectively[160]. Crizotinib is another c-MET inhibitor that has been proven to increase sensitivity to radiotherapy in patients with cetuximab-resistant KRAS-MT mCRC[161].

Rechallenge of anti-EGFR therapy

RAS mutations and EGFR ectodomain clones that appeared during anti-EGFR treatment would subside with antibody discontinuation, resulting in a decrease in the abundance of resistance mutations in the RAS/EGFR alleles, thereby restoring sensitivity to anti-EGFR regimens. However, not all patients

would benefit from the rechallenge therapy. Utilizing ctDNA is crucial for the dynamic monitoring of acquired resistance and helps to guide treatment decisions, especially for EGFR rechallenge[20]. Khan *et al*[162] found that resistance mutations can be detected in the blood a few months before disease progression. The landmark observations were from the CRICKET and CAVE clinical trials. In the CRICKET single-arm phase II study, patients treated with first-line cetuximab plus irinotecan with at least 6 mo of PFS were recruited and achieved an ORR of 21% and DCR of 54% to anti-EGFR rechallenge, respectively. All patients with PR had baseline RAS-WT, and these patients experienced longer PFS than patients with ctDNA RAS-MT (4.0 mo *vs* 1.9 mo)[163]. In CAVE phase II, single-arm clinical trial, a combination of cetuximab and avelumab has been proved to be an effective rechallenge strategy in mCRC patients with RAS-WT, and mOS reached 17.3 mo in patients with RAS/BRAF-WT ctDNA prior to anti-EGFR rechallenge[164]. The REMARRY and PURSUIT trials confirmed that baseline plasma RAS status could be a predictive indicator of rechallenge with anti-EGFR mAbs in patients with mCRC. In addition, this study demonstrated the effect of other gene mutations on the efficacy of rechallenge with anti-EGFR mAbs, including BRAF, EGFR, HER2, MET, and PIK3CA[165].

OVERCOMING RESISTANCE TO ANTI-VEGR THERAPY

Vanucizumab targets both VEGF-A and angiopoietin-2, and has manageable safety and promising anticancer effects in a phase I study[166]. Although compensatory activation of the c-MET pathway contributes to the loss of anti-VEGF drug efficacy, evidence for dual targeting of c-MET and VEGF in mCRC is rare[47]. Famitinib is a novel TKI targeting VEGFR2, PDGFRβ, and KIT[167]. In a phase II trial from China, this drug correlated with an improvement in PFS (2.8 mo *vs* 1.5 mo) and DCR (59.8% *vs* 31.4%) compared to placebo for patients with chemo-refractory mCRC[168]. In addition, the combination of bevacizumab and trifluridine/tipiracil is currently being tested in mCRC based on the promising results of preclinical and early-phase clinical trials[168,169].

TARGETING CELL CYCLE

WEE1 is a significant part of the G2/M checkpoint through its actions on CDK1 to regulate the cell cycle [170]. Adavosertib (AZD1775) is the first small-molecule inhibitor of WEE1 kinase, which has been tested in combination with chemotherapy and radiotherapy in several tumors. In addition, the protein p53 is also a pivotal factor in cell cycle arrest, inducing the inactivation of the G1/S phase checkpoint, thereby making tumor cells more dependent on the regulation of the G2/M checkpoint. Almost all tumor cells deactivate p53, most of which is mediated by TP53 mutations[171]. A randomized trial (FOCUS4-C) evaluated adavosertib in patients with KRAS and TP53-MT mCRC. Patients with both mutations who were stable or responding to chemotherapy were randomly assigned to two groups: Adavosertib and active monitoring (AM). Patients who received adavosertib showed an advantage in PFS compared to those on AM (3.61 mo *vs* 1.87 mo), although there was no significant difference in OS between the two groups (14 mo *vs* 18 mo)[172]. In addition, the combination of adavosertib and irinotecan as second-line treatment for mCRC patients with KRAS or BRAF MT is being evaluated in a phase I trial (NCT02906059). Other WEE1 inhibitors have also been explored.

OTHER TARGETS: NEUROTROPHIC TROPOMYOSIN RECEPTOR KINASE, ANAPLASTIC LYMPHOMA KINASE, AND ROS1 FUSION

Fusions of neurotrophic tropomyosin receptor kinase (NTRK) genes have been found in 1.5% of all CRCs, mainly in right-sided, MSI-H, and RAS/BRAF-WT tumors[173,174]. Larotrectinib and entrectinib, which are highly selective inhibitors of TRKA, TRKB, and TRK, were approved by the FDA in November 2018 and August 2019, respectively[175,176]. In a study by Cocco *et al*[177], larotrectinib achieved an ORR of 75%, and entrectinib resulted in an ORR of 57%. The phase II NAVIGATE trial reported that larotrectinib generated an ORR of 50%, DCR of 100%, mPFS of 5.5 mo, and mOS of 29.4 mo in ten mCRC patients[178]. Anaplastic lymphoma kinase (ALK), proto-oncogene 1, and receptor tyrosine kinase (ROS) are common driver genes in non-small cell lung cancer, but are extremely rare in mCRC, with an incidence of ≤ 1%[179,180]. In preclinical studies, the combination of crizotinib and mitomycin C appeared to have synergistic effects in CRC[181], and a series of trials on crizotinib are in progress.

IMMUNOTHERAPY

ICIs aim to improve immune surveillance and suppression by preventing tumor evasion from T-cell detection[182]. Currently, ICIs have been investigated for the treatment of multiple solid tumors with promising outcomes. Pembrolizumab, a humanized IgG4 antibody, was the first PD-1 blocker to gain FDA approval for CRC treatment in 2017. Initial data from the randomized phase III KEYNOTE-177 study showed that pembrolizumab as first-line therapy *vs* standard chemotherapy improved PFS in patients with mismatch repair deficient (dMMR) or MSI-H CRC[183]. Recently, the study published the final results: Compared with chemotherapy, pembrolizumab did not provide a profound advantage in OS because the prespecified α of 0.025 was not achieved. However, in term of mPFS, the pembrolizumab group was superior to chemotherapy group (16.5 mo *vs* 8.2 mo)[184]. Nivolumab, another humanized IgG4-based PD-1 antibody, was approved by FDA for dMMR or MSI-H CRC in the same year on the basis of CheckMate-142 trial. The ORR reached 31.1%, regardless of the tumor PD-L1 expression, and 1-year PFS and OS rates were 50.4% and 73.4%, respectively[185]. Nivolumab combined with the CTLA-4 inhibitor ipilimumab outperforms single-agent immunotherapy with acceptable adverse events. This combination regimen helped patients with dMMR or MSI-H mCRC achieve a PFS of 77% and OS of 83% at 1 year, with an ORR of 60% and DCR of 84%. Based on these data, the FDA has approved nivolumab and ipilimumab for patients with chemotherapy-refractory mCRC[186].

Patients with MMR proficient (pMMR) or MSS mCRC, which is also called "cold" tumor, could not obtain satisfactory results from ICIs; yet this subgroup accounts for the majority of CRC. Resistance mechanisms to immune checkpoint blockade in MSS mCRC include the loss of neoantigens, abnormal cell signaling, and immunosuppression[187]. Currently, strategies to improve ICIs responses in patients with pMMR and MSS CRC, such as in combination with other approaches including chemotherapy, radiotherapy, targeted agents, and other immune checkpoint modulators, are being explored[182,188,189]. For these patients, the combination of PD-1/PD-L1 and CTLA-4-blocking antibodies may have manageable safety and empowering antitumor activity[187]. In a clinical trial (NCT02754856), 21 patients with pMMR with liver-only metastases received durvalumab and tremelimumab prior to surgery, and prolonged relapse free survival (RFS) as well as T cell activation was observed[190]. Increasing evidence indicates that chemotherapy can boost the immune system by adjusting the immune microenvironment or directly stimulating antitumor responses[191]. It was suggested that 5-FU plus oxaliplatin may be the most effective chemotherapy regimen for activating PD-L1 expression and CD8 recruitment. A phase I/II study explored the safety and efficacy of durvalumab plus tremelimumab combined with FOLFOX in mCRC patients with MSS and RAS mutated status[192]. In a phase II trial, 43 patients with mCRC with MSS RAS/BRAF-WT received chemotherapy, cetuximab, and avelumab. Among them, four patients achieved complete response; the ORR and DCR were 81% and 89%, respectively[193]. Regorafenib plus nivolumab also showed an inspiring 7.9-mo median PFS for MSS mCRC in the phase Ib trial REGONIVO (NCT03406871)[194]. Radiotherapy can also promote T cell infiltration into cold tumors and has been used to improve immunotherapy efficacy[195]. A phase II study enrolled 24 patients with chemotherapy-refractory MSS mCRC who received durvalumab, tremelimumab, and radiotherapy. The results showed that the median OS was 11.4 mo, and the median PFS was 1.8 mo[196]. However, the optimal timing for the combination of immunotherapy and radiotherapy needs to be further explored. In addition, adoptive cellular therapy (ACT), as an emerging immunotherapy, has developed rapidly in several clinical studies and has been widely proven to have therapeutic efficacy in hematological malignancies. Carcinoembryonic antigen (CEA) is the most commonly researched target of chimeric antigen receptor (CAR) T cells for the treatment of mCRC. Zhang *et al*[197] recruited ten relapsed and refractory mCRC patients to evaluate the efficacy and safety of CAR-T therapy. Nearly 70% of patients achieved stable tumor control after treatment. In long-term observations, the serum CEA level decreased dramatically in most patients[197]. Moreover, the key factor to transform "cold" tumors into "hot" tumors is to stimulate immune response, and cancer vaccine is an ideal strategy. Currently, several vaccines have been studied for CRC, including autologous vaccines, dendritic cells, viral vectors, and peptide-based vaccines[198]. Relevant clinical trials are still in their early stages, and more research is needed to demonstrate their therapeutic potential[199-201].

ROLE OF TUMOR MICROENVIRONMENT

TME plasticity is a powerful driver of drug resistance. The TME contains not only the extracellular matrix (ECM), but also immune cells, endothelial cells, fibroblasts, and signaling molecules, including exosomes, chemokines, and cytokines[202]. The ECM is the main component that is remodeled during oncogenesis and progression. Collagen plays an important role in resisting tumor invasion and is regarded as a structural barrier. It was found that enriched collagen activates the PI3K/AKT signaling pathway *via* integrin α2β1 to sustain tumor growth. Notably, the inhibitor of integrin α2β1 can prevent metastasis, and the combination of integrin α2β1 inhibitor with chemotherapy showed improved curative effects[203]. In addition, the expression of type I collagen, matrix metalloproteinase (MMP-2),

and MMP-9 is upregulated in primary CRC, which participated in the degradation and regeneration of the ECM[204]. These findings will provide new ideas for the future treatment of mCRC. Fibroblasts are mesenchymal cells that secrete mitogenic growth factors such as FGF1, FGF2, hepatocyte growth factor (HGF), transforming growth factor beta-1 (TGF-β1), and TGF-β2[205]. Luraghi *et al*[206] found that HGF binds to MET receptors and stimulates MAPK and AKT to induce cetuximab resistance. This provides a theoretical basis for the concomitant blockade of FGFR and EGFR to reverse resistance to anti-EGFR mAbs. The combination of BLU9931 (an FGFR4 inhibitor) and cetuximab showed improved antitumor activity compared to cetuximab alone[207]. Exosomes exhibit potential antigenicity and can induce powerful immune responses[208]. Several studies have indicated that exosomes can be used as vaccines for mCRC. A phase I clinical trial has shown that the combination of ascites-derived exosomes (Aex) and granulocyte-macrophage colony-stimulating factor (GM-CSF) is a feasible alternative choice in the immunotherapy of mCRC[200].

WNT SIGNALING

The Wnt protein is a member of the coiled family of transmembrane receptors consisting of multiple glycoproteins and is a co-receptor for the lipoprotein receptor-related protein (LRP) family and other downstream signaling proteins[209,210]. Wnt/β-catenin signaling is the most classical pathway in Wnt signaling, and its key steps are protein stabilization and nuclear translocation. When Wnt ligands are not activated, a complex formed by adenomatous polyposis coli (APC) protein, framework protein Axin, glycogen synthase kinase 3β (GSK3β), and casein kinase 1 (CK1) will degrade βcatenin[211,212]. In the case of Wnt activation, Wnt ligands integrate with receptors, resulting in the phosphorylation of LRP by CK1 and GSK3, and recruit proteins such as axisprotein (Axin) and dishevelled to the cell membrane, thereby disrupting the formation of the complexes that could degrade βcatenin[213,214]. Undegraded β-catenin accumulates in the cytoplasm, and when it reaches a sufficient concentration, free β-catenin is transferred to the nucleus and binds to the transcription factor/lymphocyteenhancing factor (TCF/LEF), inducing cell proliferation, differentiation, and maturation[214,215]. Normal transduction of Wnt signaling facilitates the regulation of intestinal function[216,217]. Abnormal activation of Wnt/β-catenin signaling is found in more than 80% of CRCs, which leads to the accumulation of nuclear β-catenin and is associated with a poor prognosis[218]. Studies have shown that Wnt/β-catenin signaling mediates CRC resistance *via* three pathways: Tumor stem cells, non-coding RNAs (ncRNAs), and disordered TME[219-222].

β-catenin is a crucial target of Wnt signaling, thus preventing the protein-protein connection between TCF and β-catenin suppresses tumor cell growth[223]. The earliest studies utilized high-throughput enzyme-linked immunosorbent assay (ELISA) to detect the β-catenin-TCF connection, and eight compounds were initially screened, of which PKF115-584 and CGP049090 proved to be the most potent material[223]. Moreover, PKF115-584 and CGP049090 also interfere with β-catenin-APC interaction[224-226]. Another study found that 2,4-diamino-quinazoline (Table 1) exhibited solubility, metabolic stability, and oral bioavailability by preventing the β-catenin-TCF4 pathway[227,228]. Porcupine is a member of the membrane-bound O-acyltransferase (MBOAT) family. It adds a palmitoyl group to the protein, which is involved in Wnt signaling[229]. Small molecule compounds such as IWP, C59, and ETC-159 can target porcupine, thereby inhibiting the activity of the Wnt signaling pathways[230]. In addition, many natural compounds, such as curcumin, soybean-derived isoflavone-phytoestrogen genistein, and berberine, inhibit the Wnt signaling pathway by acting on different targets[231,232]. These natural compounds have the potential to treat CRC and deserve further investigation.

CONCLUSION

The heterogeneity and molecular diversity of CRC promote drug resistance and are associated with adverse outcomes. The field of CRC has continued to develop over the last few decades, leading to a better understanding of its occurrence and development. This review summarizes the currently available targeted therapies, drug resistance mechanisms, and potential treatments for mCRC. It is not difficult to find that a wider range of molecular markers is needed to assess the efficacy of targeted therapy and obtain optimal outcomes for patients. Excitingly, high-sensitivity genome sequencing methods and the application of liquid biopsy have provided deeper insight into the molecular evolution and mechanisms of resistance to treatment. In addition to the targets mentioned in this review, there are other new targets that correlate with the prognosis of patients with mCRC. The aim is to develop more specific drugs for these targets to adopt more individualized treatments and eventually improve the prognosis of patients with mCRC.

Table 1 Strategies to reverse targeted therapy resistance in clinical trials

Target(s)	Agent(s)	Phase	Condition	Treatment	Main outcomes
EGFR ECD	Sym004	Phase I	KRAS-WT mCRC	Sym004	mOS: 12.8 mo
EGFR S468R	Necitumumab	Phase II	First-line mCRC	Necitumumab + mFOLFOX6	ORR: 63.6%
$KRAS^{G12C}$	Sotorasib (AMG 510)	Phase I	Solid tumors	Sotorasib	ORR: 7.1%; DCR: 73.8%
$KRAS^{G12C}$	Adagrasib (MRTX849)	Phase II	Solid tumors	Adagrasib	ORR: 17%; DCR: 94%
BRAF	Vemurafenib	Pilot trial	Previously treated	Vemurafenib + panitumumab	DCR: 83%
BRAF	Vemurafenib	Phase I	Previously treated	Irinotecan + vemurafenib	ORR: 35%; DCR: 88%; mPFS: 7.7m
BRAF	Vemurafenib	SWOG-S1406; phase II	Previously treated	Irinotecan + cetuximab + vemurafenib	mPFS: 4.2 mo; ORR: 17%; DCR: 65%
BRAF	Encorafenib	BEACON; phase III	Previously treated	Encorafenib + binimetinib + cetuximab	mOS: 9.3; ORR: 26.8%
BRAF	Dabrafenib	Phase I/II	BRAF-WT	Dabrafenib + trametinib	ORR: 12%
HER2	Dual-targeted drugs	HERACLE A; phase II	KRAS-WT	Trastuzumab + lapatinib	mOS: 11.5 mo; mPFS: 5.0 mo; ORR: 30%
HER2	Pertuzumab; T-DM1	HERACLE B; phase II	KRAS/BRAF-WT	Pertuzumab + trastuzumab	ORR: 9.7%; mPFS: 4.1 mo
HER2	Trastuzumab pertuzumab	MyPathway; phase II	Previously treated	Trastuzumab + pertuzumab	ORR: 32%; mOS: 11.5 mo; mPFS: 2.9 mo
HER2	Trastuzumab pertuzumab	TRIUMPH;phase II	KRAS-WT	Trastuzumab + pertuzumab	ORR: 30%; mOS: 10.1 mo; mPFS: 4.0 mo
HER2	ADC	DESTINY-CRC01; phase II	Previously treated	Trastuzumab deruxtecan (T-DXd)	ORR: 45.3%; mOS: 15.5 mo; mPFS: 6.9 mo
HER2	Tucatinib	MOUNTAINEER; phase II	Previously treated	Tucatinib + trastuzumab	ORR: 52%; mOS: 18.7 mo; mPFS: 8.1 mo
MET	Tivantinib	Phase II	KRAS WT; previously treated	Tivantinib + irinotecan + cetuximab	mPFS: 8.3 mo; ORR: 44%; mOS: 19.8 mo
EGFR rechallenge	Irinotecan	CRICKET; phase II	KRAS/BRAF WT; third-line	Irinotecan + cetuximab	ORR: 21%; DCR: 54%; mOS: 9.8 mo; mPFS: 3.4 mo
EGFR rechallenge	Avelumab	CAVE; phase II	KRAS WT; third-line	Avelumab + cetuximab	mOS: 11.6 mo; mPFS: 3.6 mo; ORR: 7.8%; DCR: 65%
EGFR rechallenge	Irinotecan	REMARRY and PURSUIT; phase II	KRAS/BRAF WT	Panitumumab + irinotecan	ORR: 14%; mPFS: 3.6 mo
VEGF	Vanucizumab	Phase I	Solid tumors	Vanucizumab	DCR: 59.8%; mPFS: 2.8 mo
VEGF	Bevacizumab	TRIBE; phase II	mCRC	Bevacizumab + FOLFOXIRI	mPFS: 12.1 mo; mOS: 30.9 mo
VEGF	Aflibercept	VALOUR; phase III	Previously treated	Aflibercept + FOLFIRI	mOS: 13.5 mo; mPFS: 6.9 mo
VEGF	Aflibercept	AFFIRM; phase II	First-line	Aflibercept + FOLFOX	mPFS: 8.48 mo; 12mPFS: 25.8%
VEGF	Ramucirumab	RAISE; phase III	Second-line	Ramucirumab + FOLFIRI	mOS: 13.3 mo
VEGF	Bevacizumab	C-TASK FORCE; phase I/II	Previously treated	Bevacizumab + trifluridine/tipiracil	16 wk PFS: 46.9%
TKI	Famitinib	Phase II	Previously treated	Famitinib	mPFS: 2.8 mo; mOS: 7.4 mo; DCR: 59.8%; ORR: 2.2%
NTRK	Larotrectinib	NAVIGATE; phase I/II	Solid tumors	Larotrectinib	ORR: 50%; DCR: 100%; mPFS: 5.5 mo;

					mOS: 29.4 mo
PD-1/PD-L1	Pembrolizumab	KEYNOTE-177; phase III	dMMR/MSI-H CRC; first-line	Pembrolizumab	mPFS: 16.5 mo; mOS: NA
PD-1/PD-L1	Nivolumab	CheckMate-142; phase II	dMMR/MSI-H CRC; previously treated	Nivolumab	ORR: 60%; DCR: 84%
PD-1/PD-L1	Durvalumab	Phase I	Solid tumors	Durvalumab + tremelimumab	DCR: 36.4%
PD-1/PD-L1	Durvalumab	Phase Ib/II	MSS RAS/BRAF WT	Durvalumab + tremelimumab + FOLFOX	ORR: 81%; DCR: 89%
PD-1/PD-L1	Nivolumab	Phase Ib	MSS	Nivolumab + regorafenib	mPFS: 7.9 mo
PD-1/PD-L1	Durvalumab	Phase II	Chemotherapy-refractory MSS	Durvalumab + tremelimumab + radiotherapy	ORR: 8.3%; mPFS: 1.8 mo; mOS: 11.4 mo

mCRC: Metastatic colorectal cancer; EGFR: Epidermal growth factor receptor; VEGF: Vascular endothelial growth factor; TKI: Tyrosine kinase inhibitors; ADC: Antibody-drug conjugate; ORR: Overall response rate; mOS: Median overall survival; mPFS: Median progression-free survival; DCR: Disease control rate; PD-1: Programmed cell death protein 1; PD-L1: Programmed death-ligand 1; MSS: Microsatellite stable; FOLFOX: Leucovorin, 5-fluorouracil, and oxaliplatin; FOLFIRI: Leucovorin, 5-fluorouracil, and irinotecan; T-DXd: Trastuzumab deruxtecan; HER: Human epidermal growth factor receptor.

FOOTNOTES

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Country/Territory of origin: China

ORCID number: Xin Wang 0000-0003-3599-6904.

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REVIEW

Decade of optimizing therapy with direct-acting antiviral drugs and the changing profile of patients with chronic hepatitis C

Michał Brzdęk, Dorota Zarębska-Michaluk, Federica Invernizzi, Marta Cilla, Krystyna Dobrowolska, Robert Flisiak

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Michał Brzdęk, Krystyna Dobrowolska, Collegium Medicum, Jan Kochanowski University, Kielce 25-516, Poland

Dorota Zarębska-Michaluk, Department of Infectious Diseases, Jan Kochanowski University, Kielce 25-516, Poland

Federica Invernizzi, Marta Cilla, Center for Liver Disease, Division of Internal Medicine and Hepatology, IRCCS Ospedale San Raffaele, Milan 20-132, Italy

Robert Flisiak, Department of Infectious Diseases and Hepatology, Medical University of Białystok, Białystok 15-540, Poland

Corresponding author: Dorota Zarębska-Michaluk, PhD, Professor, Department of Infectious Diseases, Jan Kochanowski University, Radiowa 7, Kielce 25-516, Poland. dorota1010@tlen.pl

Abstract

Chronic infection with the hepatitis C virus (HCV) remains a major health problem affecting approximately 58 million people worldwide. In the era of interferon (IFN)-based regimens, patients particularly infected with genotypes 1 and 4 achieved a low response rate. The implementation of direct-acting antivirals changed the landscape of HCV treatment. The increase in effectiveness provided us with the hope of eliminating HCV as a significant public threat by 2030. In the following years, there was an observed improvement in the treatment of HCV with genotype-specific regimens and highly effective pangenotypic options that are the most recent stage of the revolution. The optimization of therapy was accompanied by changes in the patient profile from the beginning of the IFN-free era over time. Patients treated with antiviral therapies were younger in successive periods, less burdened with comorbidities and comedications, more frequently treatment-naïve and had less advanced liver disease. Before the IFN-free era, specific subpopulations such as patients with HCV/HIV coinfection, those with a history of previous treatment, patients with renal impairment or with cirrhosis had lower chances for a virologic response. Currently, these populations should no longer be considered difficult to treat. Despite the high effectiveness of HCV therapy, there is a small percentage of patients with treatment failure. However, they can be effectively retreated with pangenotypic rescue regimens.

Key Words: Hepatitis C virus; Interferon; Direct-acting antiviral; Epidemiology; Chronic

hepatitis C

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Core Tip: A decade ago direct-acting antiviral drugs were introduced and have greatly improved the effectiveness of treatment for patients with hepatitis C virus infection. The new drugs have increased the chances of eliminating hepatitis C virus infection as a significant public threat. This paper described changes in the therapeutic options administered over the past decade and documented the changing patient profile over the years in the interferon-free era. Special populations of difficult-to-treat patients in the interferon era currently not meeting this definition are highlighted.

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INTRODUCTION

Chronic infection with hepatitis C virus (HCV) is one of the leading causes of liver disease in the world. According to the most recent estimates from the World Health Organization (WHO), 58 million people are living with chronic hepatitis C (CHC) worldwide, with the annual number of new infections reaching 1.5 million[1]. About 20% of them are at risk of developing liver cirrhosis, eventually leading to organ failure and hepatocellular carcinoma[2]. These most severe complications of the disease are the cause of death of approximately 290000 people a year. There is no effective vaccine against HCV, but currently available direct-acting antivirals (DAA) can cure more than 95% of people infected with hepatitis C regardless of the genotype (GT).

To date, six major HCV GTs have been identified that differ in more than 35% of the genome sequence[3]. The most common of these worldwide is GT1, considered the most difficult to treat in the era of interferon (IFN)-based therapies. However, this was the first GT in which very high efficacy was achieved with GT-specific therapies at the beginning of the DAA era[4]. Further evolution of antiviral therapy has led to the availability of regimens that are highly effective in infection with any HCV GT and at any stage of liver disease, including decompensated liver cirrhosis[5]. In addition to increased efficacy, safety parameters have improved, and antiviral therapy has become shorter and now lasts 8 or 12 wk in the vast majority of patients[6]. There was also a simplification of regimens by dropping the addition of ribavirin (RBV), which also translated into improved tolerability. Optimization of antiviral therapy has set the groundwork for achieving the WHO's goal of eliminating HCV infection as a public health threat by 2030. The goal to realize this target remains the diagnosis of infection in patients who are unaware of it.

CHANGES IN THERAPEUTIC REGIMENS

IFN-based therapy

Regimens without DAA: The era of IFN in the treatment of HCV infection began in 1986, even before the identification of the virus, which was then known as non-A, non-B hepatitis virus. Hoofnagle *et al*[7] documented a reduction in transaminase activity as a result of IFNα2b therapy. Despite the beneficial effect of reducing disease activity, the initial responses to treatment were transient[8]. In subsequent years, other investigators reported that the decline was related to viral clearance or a decrease in serum HCV RNA[9]. Sustained virological response (SVR) after 24-wk monotherapy with recombinant IFN ranged from 3%-6%, and IFN administered for 48 wk resulted in 13%-19% efficacy[10,11]. Adding RBV to IFN improved treatment efficacy to about 40%[10-13].

The next milestone in the antiviral treatment was the introduction of two forms of long-acting pegylated IFN (pegIFN), alfa-2a and alfa-2b, which were used once a week. This change was one of the key elements in improving treatment adherence and greater efficacy of antiviral therapy[14,15]. In randomized multicenter trials, the SVR rate with pegIFN monotherapy ranged from 15% to 39%[16,17], and the addition of RBV to the regimen increased efficacy to about 50%[15,18]. HCV GTs has been identified as the most important independent predictor of response to pegIFN + RBV treatment.

The prospective PROPHESYS study involving 7163 patients treated with pegIFN + RBV for 48 wk showed better efficacy rates of 71.4% and 60.6% in patients with GT2 and GT3 infections, respectively, compared to patients with GT1 (41.8%) and GT4 (41%) infections. The higher efficacy of therapy was also observed in patients without advanced liver fibrosis or cirrhosis[19]. The most important predictor of response to therapy regardless of HCV GT was undetectable HCV RNA at week 4 of therapy, defined as the rapid virological response as proven by a retrospective study involving 1383 patients[20]. These findings allowed the reduction of the length of treatment to less than 24 wk in GT2- and GT3-infected patients who achieved rapid virological response without a significant decrease in efficacy compared to those with detectable HCV RNA after 4 wk of treatment who required treatment for 24 wk[21]. In addition to virologic response at week 4, treatment length was determined by the response at week 12; GT1-infected patients with detectable serum HCV RNA after 12 wk of treatment benefited from extending treatment to 72 wk[22,23].

The efficacy of IFN-containing regimens was limited by safety issues; adverse events of therapy were frequent and led to treatment discontinuation in 5%-16% of cases[15,24]. Fatigue, headache, fever, malaise, myalgia, neutropenia and thrombocytopenia were reported as the most common adverse events[25]. The main side effect associated with RBV use resulting in its dose reduction in 20% of patients was hemolytic anemia[18].

Regimens with DAA: The introduction of the protease inhibitors telaprevir (TVR) and boceprevir in 2011 launched the era of the first DAAs for the treatment of HCV infection[26,27]. Initially, the new drugs were registered for use with pegIFN + RBV only in patients with GT1 infection. Cure rates following triple therapy increased to about 70% compared to previously used regimens containing only pegIFN + RBV[28]. Results from the randomized, double-blind, placebo-controlled ADVANCE trial evaluating a triple regimen with TVR in treatment-naïve GT1-infected patients showed that therapy could be shortened to 24 wk in most without reducing efficacy[29].

Among patients after previous treatment failure assessed in the randomized phase 3 REALIZE trial, patients with prior relapse achieved SVR rates of 83%-88% following treatment with pegIFN + RBV + TVR, while this was only 30% for null responders[30].

The most difficult population to treat with a triple regimen containing first-generation protease inhibitors, TVR or boceprevir, was the population of treatment-experienced patients with liver cirrhosis due to very low efficacy and a poor safety profile[31-33].

Registration of new DAAs has broadened the spectrum of clinical use of triple-drug regimens. The protease inhibitor simeprevir and the non-structural protein 5A (NS5A) inhibitor daclatasvir (DCV) combined with pegIFN + RBV could be used in GT1-infected patients and those with GT4 infection with an SVR rate exceeding 82% in treatment-naïve patients[34-36]. The addition of the polymerase inhibitor, sofosbuvir (SOF) to pegIFN + RBV enabled triple therapy for patients infected with any HCV GT[37,38].

IFN-free regimens

GT-specific options: Although triple-drug therapies have resulted in higher SVR rates compared to pegIFN + RBV treatment, there is still a major limitation related to side effects as well as contraindications to therapy when using IFN. Patients with liver cirrhosis and hematologic disorders as well as those with decompensated liver disease or severe diseases of other organs and systems were left out of treatment. These safety issues were overcome by the introduction of IFN-free therapies. These well-tolerated and highly effective regimens revolutionized the antiviral therapy of CHC[39]. The first registered DAA that could be used without IFN was SOF[37]. The combination of this drug with the inhibitor of HCV NS5A protein, ledipasvir (LDV), administered with or without RBV resulted in a GT-specific combination active against GT1 and 4 infections.

Randomized, open-label phase 3 trials, ION-1 and ION-2, documented 95%-100% efficacy in GT1-infected patients depending on treatment duration (12 or 24 wk), prior treatment history and RBV use. However, lower efficacy was observed in treatment-experienced patients with liver cirrhosis[40,41] (Table 1). The ION-3 study demonstrated that in treatment-naïve patients without liver cirrhosis even shortening SOF/LDV ± RBV therapy to 8 wk does not significantly affect efficacy[42].

The results of a retrospective real-world evidence (RWE) study conducted in a large cohort of patients in the United States confirmed the high effectiveness of this regimen with 93% of GT1-infected patients responding in 93% (n = 9912), while the cure rate in GT4-infected patients was 87% (n = 97)[43].

Another GT-specific option registered for patients with compensated liver disease was the combination of ombitasvir (OBV), an inhibitor of NS5A, and ritonavir-boosted paritaprevir (PTV/r), an HCV protease inhibitor. In cases of infection with GT4, it was used with RBV for 12 wk, while for GT1-infected patients the addition of a third DAA, dasabuvir (DSV), acting as a polymerase inhibitor, was obligatory[44]. The addition of RBV and the length of therapy depended on the subtype and the presence of liver cirrhosis.

The phase 3 clinical trials SAPPHIRE-I and SAPPHIRE-II confirmed the efficacy of the 12-wk option of OBV/PTV/r + DSV + RBV at the level of 96% in GT1-infected patients without liver cirrhosis regardless of history of previous therapy[45,46]. Results from the phase 3 PEARL-III and PEARL-IV clinical trials proved that the inclusion of RBV improved efficacy in GT1a-infected patients, while the SVR in GT1b-infected patients exceeds 99% regardless of RBV addition[47].

Table 1 Efficacy of genotype-specific regimens in phase III of clinical trials

Ref.	Number of participants	Regimen	GT	SVR (mITT analysis) *n*/*N* (%)			
				Patients without cirrhosis		Patients with cirrhosis	
				Treatment-naïve	Treatment-experienced	Treatment-naïve	Treatment-experienced
Afdhal *et al*[40] (ION-1)	865	LDV/SOF, 12 wk (*n* = 214)	1	179/179 (100)		32/33 (97.0)	
		LDV/SOF + RBV, 12 wk (*n* = 217)		178/178 (100)		33/33 (100)	
		LDV/SOF, 24 wk, (*n* = 217)		181/182 (99.5)		31/32 (96.9)	
		LDV/SOF + RBV, 24 wk (*n* = 217)		179/179 (100)		36/36 (100)	
Afdhal *et al*[41] (ION-2)	440	LDV/SOF, 12 wk (*n* = 109)	1		83/87 (95.4)		19/22 (86.4)
		LDV/SOF + RBV, 12 wk (*n* = 111)			89/89 (100)		18/22 (81.8)
		LDV/SOF, 24 wk, (*n* = 109)			86/87 (98.9)		22/22 (100)
		LDV/SOF + RBV, 24 wk (*n* = 111)			88/89 (98.9)		22/22 (100)
Kowdley *et al*[42] (ION-3)	647	LDV/SOF, 8 wk (*n* = 215)	1	202/214 (94.4)			
		LDV/SOF + RBV, 8 wk (*n* = 216)		201/211 (95.3)			
		LDV/SOF, 12 wk (*n* = 216)		206/209 (98.6)			
Feld *et al*[45] (SAPPHIRE-I)	473	OBV/PTV/r + DSV + RBV, 12 wk	1	455/473 (96.2)			
Zeuzem *et al*[46] (SAPPHIRE-II)	297	OBV/PTV/r + DSV + RBV, 12 wk	1		286/297 (96.3)		
Ferenci *et al*[47] (PEARL-III)	419	OBV/PTV/r + DSV + RBV, 12 wk (*n* = 210)	1b	209/210 (99.5)			
		OBV/PTV/r + DSV + placebo, 12 wk (*n* = 209)		207/207 (100)			
Ferenci *et al*[47] (PEARL-IV)	305	OBV/PTV/r + DSV + RBV, 12 wk (*n* = 100)	1a	97/99 (98.0)			
		OBV/PTV/r + DSV + placebo, 12 wk (*n* = 205)		185/204 (90.7)			
Welzel *et al*[48] (GARNET)	163	OBV/PTV/r + DSV, 8 wk	1b	160/162 (99.0)			
Asselah *et al*[49] (AGATE-I)	120	OBV/PTV/r + RBV, 12 wk (*n* = 59)	4			57/58 (98.0)	
		OBV/PTV/r + RBV, 16 wk (*n* = 61)				60/60 (100)	
Asselah *et al*[50] (AGATE-I Part II)	64	OBV/PTV/r + RBV, 24 wk	4			60/60 (100)	
Waked *et al*[51] (AGATE-II)	160	OBV/PTV/r + RBV, 12 wk (*n* = 131)	4	94/98 (96.0)	30/31 (97.0)		
		OBV/PTV/r + RBV, 24 wk (*n* = 29)				27/28 (96.0)	
Zeuzem *et al*[58] (C-EDGE treatment naïve)	421	GZR/EBR ± RBV, 12 wk	1, 4, 6	68/70 (97.0)[1]		231/246 (94.0)[1]	
Kwo *et al*[59] (C-EDGE treatment-experienced)	420	GZR/EBR ± RBV, 12 or 16 wk	1, 4, 6		255/264 (96.6)		135/144 (93.8)

[1]Intent-to-treat analysis (no data for modified intent-to-treat analysis).
DSV: Dasabuvir; EBR: Elbasvir; GT: Genotype; GZR: Grazoprevir; LDV: Ledipasvir; mITT: Modified intention-to-treat; OBV: Ombitasvir; PTV/r: Paritaprevir; RBV: Ribavirin; SOF: Sofosbuvir; SVR: Sustained virological response.

The findings of the phase 3b study GARNET allowing the shortening of OBV/PTV/r + DSV therapy in GT1b-infected patients without cirrhosis to 8 wk without affecting efficacy formed the basis for updating the label[48]. Patients with GT4 infection both with and without compensated cirrhosis treated with OBV/PTV/r + RBV in the multinational AGATE-I and AGATE-I part II trials and the Egyptian AGATE-II trial achieved high SVR rates of 96%-100%. The demonstration that prolonging therapy did not improve the success rate among patients with compensated cirrhosis became the basis for updating the label, and regardless of the severity of liver disease, GT4-infected patients received a 12-wk treatment course[49-51]. The high effectiveness of the OBV/PTV/r + DSV +/- RBV regimen of 95% to 100% in GT1 and GT4-infected patients regardless of the severity of liver disease[52-54] and history of prior antiviral treatment has been confirmed in numerous real-world studies[55-57].

Another GT-specific DAA regimen designed for patients with GT1 and GT4 infection without cirrhosis and with compensated cirrhosis includes combinations of the protease inhibitor, grazoprevir (GZR), and the NS5A inhibitor, elbasvir (EBR), used for 12 wk. The option to add RBV and extend therapy to 16 wk applies to GT1a and GT4-infected patients with an initial viral load > 800000 IU/mL. Efficacy in phase 3 clinical trials in treatment-naïve patients without cirrhosis and those with compensated cirrhosis was 97% and 94%, respectively, while treatment-experienced patients responded in 96.6% and 93.8%, respectively[58,59]. However, it should be emphasized that these are data calculated for intent-to-treat analysis.

Pangenotypic options: The first available DAA regimen with activity against all HCV GTs was a combination of SOF and RBV. The efficacy of 12 wk of therapy with SOF + RBV in treatment-naïve patients with GT1-3 infection evaluated in a phase 3 clinical trial was 72% in patients without cirrhosis and 47% in patients with cirrhosis, respectively[60] (Table 2). A higher response rate of 97.1% was documented in patients with GT2 infection. The efficacy of this regimen in treatment-experienced GT2 and GT3-infected patients participating in the phase 3 FUSSION study was 96% *vs* 37% for patients without cirrhosis and 60% *vs* 19% in patients with cirrhosis, respectively[61]. Extending the duration of therapy to 16 wk increased the cure rates to 100% and 62.5% in patients without cirrhosis infected with GT2 *vs* GT3, while patients with liver cirrhosis responded in 62.5% and 61.0%, respectively. A comparison of treatment duration (16 wk *vs* 24 wk) in patients with GT3 infection documented the higher efficacy followed longer therapy with SVR of 86.5% and 78.6% in patients without cirrhosis and those with cirrhosis, respectively[62].

Extending treatment to 24 wk improved the response rate, especially in the GT3-infected treatment-experienced patients with cirrhosis responding in 76%. These values were still unsatisfactory, and the regimen was considered suboptimal in this patient population[62,63,64].

The use of a 12-wk regimen with pangenotypic activity, consisting of SOF and DCV, improved SVR rates in GT3-infected patients without cirrhosis to about 97% in treatment-naïve individuals and 94% in those who failed prior therapy. However, the efficacy reported in patients with cirrhosis was much lower, approximately 63%[65].

A real breakthrough in the treatment of CHC came with the introduction of new pangenotypic options highly effective against all HCV GTs. Based on phase 3 ASTRAL registration studies, in 2016 the Food and Drug Administration approved a combination consisting of SOF and the NS5A inhibitor velpatasvir (VEL) for patients without and with liver cirrhosis, including decompensated individuals [66]. The 12-wk regimen in patients with compensated liver disease resulted in an SVR of 95%-100% regardless of the history of previous therapy, while patients with decompensated liver cirrhosis responded in 84%[67-69]. The extension of therapy to 24 wk in this patient population increased the response rate to 88% and the addition of RBV to 94% in the modified intent-to-treat analysis[69]. The lowest efficacy was achieved in GT3-infected patients, with SVR rates ranging from 50% to 85%, but it should be noted that the number of patients in this study was relatively small, 12-14 in each treatment arm.

The high efficacy of SOF/VEL was confirmed in a meta-analysis of 12 studies from routine clinical practice. Of the 5196 patients with response rate data, 99% achieved SVR[70]. The analysis revealed unfavorable factors associated with a reduction in the odds of virologic response to about 98%, such as GT3 and compensated liver cirrhosis. Importantly, patients with current or prior decompensated cirrhosis were excluded from the analysis. Another new pangenotypic option containing the protease inhibitor glecaprevir (GLE) and the NS5A inhibitor pibrentasvir (PIB) became available for patients with compensated liver disease after approval by Food and Drug Administration in 2017[71].

The efficacy of the 12-wk GLE/PIB regimen in patients with GT 1, 2, 4-6 infection without cirrhosis as well as with compensated cirrhosis participating in phase 3 clinical trials reached 100% and 99%, respectively[72-74]. An equally high efficacy of 99% was reported in GT3-infected patients without liver cirrhosis in the phase 3 ENDURANCE-3 trial[72].

Table 2 Efficacy of pangenotypic regimens in phase III of clinical trials

Ref.	Number of participants	Regimen	GT	SVR (mITT analysis) *n*/*N* (%)			
				Patients without cirrhosis		Patients with cirrhosis	
				Treatment-naïve	Treatment-experienced	Treatment-naïve	Treatment-experienced
Lawitz *et al*[60] (FISSION)	256	SOF + RBV, 12 wk	1-3	147/204 (72.1)		23/49 (46.9)	
Jacobson *et al*[61] (FUSION)	201	SOF + RBV, 12 wk (*n* = 103)	2, 3		25/26 (96.2), 14/38 (36.8)		6/10 (60.0), 5/26 (19.2)
		SOF + RBV, 16 wk (*n* = 98)	2, 3		23/23 (100), 25/40 (62.5)		7/9 (77.8), 14/23 (60.9)
Jacobson *et al*[61] (POSITION)	207	SOF + RBV, 12 wk	2		85/92 (92.0%)		16/17 (94.0%)
			3		57/84 (68.0%)		3/14 (21.0%)
Zeuzem *et al*[81] (VALENCE)[1]	323	SOF + RBV, 12 wk (*n* = 73)	2	29/30 (96.7)	30/32 (93.8)	2/2 (100)	7/9 (77.8)
		SOF + RBV, 24 wk (*n* = 250)	3	87/92 (94.6)	85/98 (86.7)	12/13 (92.3)	29/47 (61.7)
Omata *et al*[82]	153	SOF + RBV, 12 wk	2	80/82 (95.0)	52/54 (100)	8/8 (100)	8/9 (89.0)
Foster *et al*[62] (BOSON)[1]	363	SOF + RBV, 16 wk	3	58/70 (83.0)	41/54 (76.0)	12/21 (57.0)	17/36 (47.0)
		SOF + RBV, 24 wk		65/72 (90.0)	44/54 (81.0)	18/22 (82.0)	26/34 (76.0)
Satsangi *et al*[83]	105	SOF + RBV, 24 wk	3	49/49 (100)	1/1 (100)	22/23 (95.6)	3/3 (100)
Nelson *et al*[65] (ALLY-3)	152	DCV + SOF, 12 wk	3	105/109 (96.0)		20/32 (63.0)	
				73/75 (97.0)	32/34 (94.0)	11/19 (58.0)	9/13 (69.0)
Feld *et al*[67] (ASTRAL-1)	624	SOF/VEL, 12 wk	1, 2, 4, 5, 6	496/501 (99.0)		120/121 (99.2)	
Foster *et al*[68] (ASTRAL-2)	266	SOF/VEL, 12 wk (*n* = 134)	2	133/134 (99.0)			
		SOF + RBV, 12 wk (*n* = 132)		124/132 (94.0)			
Foster *et al*[68] (ASTRAL-3)	552	SOF/VEL, 12 wk (*n* = 277)	3	160/163 (98.0)	31/34 (91.0)	40/43 (93.0)	33/37 (89.0)
		SOF + RBV, 24 wk (*n* = 275)		141/156 (90.0)	22/31 (71.0)	33/45 (73.0)	22/38 (58.0)
Curry *et al*[69] (ASTRAL-4)	267	SOF/VEL, 12 wk (*n* = 90)	1-6			75/89 (84.3)	
		SOF/VEL + RBV 12 wk (*n* = 87)				82/87 (94.0)	
		SOF/VEL, 24 wk (*n* = 90)				77/87 (88.5)	
Forns *et al*[73] (EXPEDITION-1)	146	GLE/PIB, 12 wk	1, 2, 4, 5 or 6			145/146 (99.0)	
Zeuzem *et al*[81] (ENDURANCE-1)	703	GLE/PIB, 8 wk (*n* = 351)	1	343/344 (99.7)			
		GLE/PIB, 12 wk (*n* = 352)		345/345 (100)			
Zeuzem *et al*[72] (ENDURANCE-3)	505	GLE/PIB, 12 wk (*n* = 233)	3	214/217 (99.0)			
		SOF + DCV, 12 wk (*n* = 115)		110/111 (99.0)			
		GLE/PIB, 8 wk (*n* = 157)		143/149 (96.0)			
Asselah *et al*[74]	202	GLE/PIB, 12 wk	2	192/192 (100)			

(ENDURANCE-2)					
Asselah *et al*[74] (ENDURANCE-4)	121	GLE/PIB, 12 wk	4-6	120/120 (100)	
Brown *et al*[76] (EXPEDITION-8)	343	GLE/PIB, 8 wk	1-6		334/335 (99.7)
Gane *et al*[78] (EXPEDITION-4)	104	GLE/PIB, 12 wk	1-6	102/104 (98.0)	
Bourlière *et al*[84] (POLARIS-1)[1]	263	SOF/VEL/VOX, 12 wk	1-6	253/263 (96.0)[2]	
Bourlière *et al*[84] (POLARIS-4)[1]	182	SOF/VEL/VOX, 12 wk	1-4	178/182 (98.0)[2]	

[1]Intent-to-treat analysis (no data for modified intent-to-treat analysis).

[2]All analyzed patients were previously treated with a direct-acting antiviral-containing regimen.

DCV: Daclatasvir; GLE: Glecaprevir; GT: Genotype; mITT: Modified intention-to-treat; PIB: Pibrentasvir; RBV: Ribavirin; SOF: Sofosbuvir; SVR: Sustained virological response; VEL: Velpatasvir; VOX: Voxilaprevir.

Shortening therapy to 8 wk in patients infected with GT1-6 without cirrhosis evaluated in the SURVEYOR-I and -II phase 2 studies and with compensated cirrhosis participating in the EXPEDITION-8 phase 3 trial did not affect the efficacy of therapy and became the basis for updating the label[75,76]. However, the EudraCT trial showed that further reduction of therapy to 4 wk reduced efficacy to 59% (10/17) for GLE/PIB without RBV and to 73% (11/15) for GLE/PIB + RBV, and drug target-specific substitutions associated with NS5A resistance were identified as a predictor of virologic failure[77].

The registration of the GLE/PIB option for use in GT1-6-infected patients with stage 4 or 5 chronic kidney disease, including those on dialysis, was based on the results of the EXPEDITION-4 trial with a 98% SVR rate in this population of patients[78]. The high efficacy of treatment with the GLE/PIB regimen has been confirmed in everyday clinical practice. A meta-analysis of 18 RWE studies involving 12531 patients documented greater than 95% efficacy regardless of GT, presence of compensated cirrhosis and history of prior therapy[79].

In 2017, a new triple pangenotypic regimen containing SOF/VEL and the HCV protease inhibitor voxilaprevir (VOX) was also registered for patients with compensated liver disease[80]. Despite its high efficacy regardless of the history of previous therapy, in practice it is used primarily as a rescue regimen after the failure of DAA therapy, and it is discussed in detail later in this manuscript, in the section on rescue therapy.

CHANGES IN THE PROFILE OF HCV-INFECTED PATIENTS OVER TIME

The availability of safe and effective therapy has led the WHO to set a goal of eliminating HCV as a significant public threat by 2030. This goal was defined as a 65% reduction in mortality and a 90% reduction in new chronic infections compared to 2015[85]. In June 2021, the WHO presented targets to reduce the annual incidence to ≤ 5 per 100000 and annual mortality to ≤ 2 per 100000[86]. In a large model study representing 93% of the global population, the Polaris Observatory HCV Collaborators estimated a reduction in global HCV prevalence from 0.9% (63.6 million) in 2015 to 0.7% (56.8 million) in 2020. During the analysis period, 8.7 million patients worldwide were treated, and significant changes were observed in the demographic and clinical characteristics of HCV-infected patients[87].

Published data indicated that patients treated with antiviral therapy for chronic HCV infection were less burdened by comorbidities and were less likely to use comedications in the subsequent time intervals analyzed[88-91]. A decrease in the median age of treated patients was also observed. Elsharkawy *et al*[88] analyzed the data of 337042 Egyptian patients of the national CHC treatment program who started therapy from October 2014 (the first introduction of IFN-free regimens in Egypt) to March 2016. The population was divided into three-time intervals of 6 mo each. The results showed a reduction in age (mean ± SD) from 53 ± 9 years in the first cohort to 49 ± 12 years in the last group[88,92]. In the Polish cohort of 10152 patients observed between 2015 and 2018, a more pronounced reduction in median age was observed from 58 at the beginning of the analyzed period to 49 at the end [89]. Furthermore, the prevalence of comorbidities and the use of concomitant medications decreased significantly in subsequent time intervals from 68.6% to 59.5% and from 64.6% to 57.7%, respectively [89].

A study carried out in the United States documented a 9.3% increase in the proportion of HCV RNA-positive patients under the age of 50 between 2017 and 2019, while German observations covering the period 2014-2018 demonstrated an increase of 14.7% in this age group of treated patients[90,91].

In 2015, Messina *et al*[3] published a comprehensive study on the prevalence and distribution of GTs worldwide before the IFN-free era. The analysis included 1217 studies published between 1993 and 2013, and they documented GT1 as the most common HCV GT globally (46%), followed by GT3 and GT4 (30% and 9%, respectively). The prevalence of GTs showed geographic variation; the proportion of GT4 in central sub-Saharan Africa accounted for more than 97% of all HCV infections. At the end of 2015, the estimated order of GTs remained the same, with GT1 and GT3 being the dominant GTs worldwide at 44% and 25% prevalence, respectively, and GT4 continuing to rank third with a 15% prevalence[93]. Given the high efficacy of pangenotypic regimens, the need for genotyping seems to be losing ground[94], and to the best of our knowledge, no studies have been published in the last few years on the prevalence and distribution of GTs at the global level.

However, several papers have analyzed this issue in patient populations at the national level. Studies of Polish and German cohorts have shown a high proportion of GT1-infected patients among antiviral-treated patients at the beginning of the DAA era[89,95]. Over time, the proportion of these patients has declined in favor of GT3-infected patients. The explanation for this phenomenon is related to the changes in therapeutic regimens discussed above. At the beginning of the DAA era, GT1-infected patients had access to highly effective GT-specific regimens, while for patients with GT3 infection there was only one IFN-free option, the 24-wk SOF + RBV combination. Because of the suboptimal virologic response achieved with this regimen, many GT3-infected patients, especially those with non-advanced liver disease, preferred the shorter 12-wk triple-drug treatment of SOF + pegIFN + RBV or waiting for more effective IFN-free options[63]. Another IFN-free therapeutic option that was more effective in patients with GT3 infection, the SOF + DCV + RBV combination, was not widely available in many countries since it contained drugs from different manufacturers, and this made registration and reimbursement difficult.

Thus the prevalence of GT1-infected DAA-treated patients decreased by 9% in Poland between 2015 and 2018 and 12% in Germany between 2014 and 2019. A similar trend was observed among 76110 patients after the introduction of DAA regimens in China, with the percentage of GT3-infected patients increasing by almost 6% between 2016 and 2020[96].

At the beginning of the IFN-free era, the proportion of patients with advanced fibrosis and cirrhosis was significantly higher compared to subsequent years, likely related to priority access to reimbursable DAA options in this patient population[97]. A single-center RWE study of Polish patients documented that the percentage of patients on antiviral therapy who were diagnosed with cirrhosis decreased significantly from 51% in 2015-2016 to 11.8% in 2018[98].

SPECIFIC SUBPOPULATIONS OF HCV-INFECTED PATIENTS

In the era of IFN-based antiviral therapies, specific subpopulations of patients were identified and recognized as difficult to treat because of more prevalent contraindications to therapy, poorer adherence, more frequent adverse events with associated treatment discontinuation and lower efficacy [4,25]. In the era of DAA therapy, these populations are essentially no longer difficult to treat, although some researchers have noted factors that negatively affect the success of therapy, especially if they occur together.

Patients with HCV/HIV coinfection

The prevalence of HCV/HIV coinfection varies significantly across subpopulations and geographic regions. Recent estimates have shown that about 2.5% of HIV-infected patients are coinfected with HCV [99]. In the era of IFN-based regimens, HIV/HCV coinfected patients were considered more challenging compared to those with HCV monoinfection. Inferior adherence, lower SVR rates, drug interactions and unfavorable safety profiles were significant therapeutic concerns[100]. DAA therapy has revolutionized the treatment of HIV/HCV-coinfected individuals, improving adherence, efficacy and safety profile. Clinical trials using a range of DAA options have shown high rates of SVR exceeding 95% and comparable to efficacy in patients with HCV monoinfection regardless of the type of regimen used[101-104]. Similar high effectiveness has been observed in studies carried out in routine medical practice, although some authors have reported lower response rates to DAA therapy in populations with HIV coinfection than in populations with HCV monoinfection[105-109].

Treatment-experienced patients

Similar observations apply to patients with a history of prior failed therapy. Numerous clinical trials with different DAA options do not document the lower SVR rates as compared to treatment-naïve patients, while in some RWE studies this parameter has been identified as an unfavorable factor in the chances of virologic response with lower SVR rates ranging from 0.7% to 2.4% depending on the regimen used, type of GT and severity of liver disease[39,55,70,73,110].

A distinct category of treatment-experienced patients is those who have not responded to IFN-free options subjected to subsequent DAA therapy. Data from the German hepatitis C registry described an unsatisfactory 79.5% response rate in patients retreated with DAA after the failure of initial IFN-free

treatment[111]. These results were supported by findings from a Polish RWE study reporting effectiveness of 86% with GT-specific DAA retreatment in previous IFN-free nonresponders[112]. A newly published analysis also from Poland evaluated patients who failed pangenotypic therapy and underwent pangenotypic retreatment[113].

Patients with renal impairment

Chronic infection with HCV may be an independent risk factor for the development or progression of chronic kidney disease (CKD)[114]. On the other hand, due to suboptimal hygiene measures in some dialysis units, the prevalence of HCV infection in hemodialysis patients was significantly higher (3.8%-31.1%)[115]. Historically, the use of IFN + RBV therapy in patients with renal failure has been limited due to contraindications to both medications[116]. Also, the efficacy of the IFN-based regimen in this patient population was lower and the safety profile was inferior compared to patients with normal renal function. Hemodialyzed patients with CHC treated with pegIFN analyzed in a meta-analysis of 24 clinical trials achieved an SVR rate of 40%, while 14% of them discontinued treatment due to adverse events[117]. Due to the distinct metabolic pathways of DAAs, their introduction to treatment has offered an effective and well-tolerated therapy for patients with renal impairment, including hemodialysis patients with HCV infection[118].

SOF-containing regimens are approved for use in patients with estimated glomerular filtration rate > 30 mL/min/1.73 m^2 due to limited safety data in patients with severe renal impairment. However, since 2019 according to the label, these combinations can also be administered with no dose adjustment at lower estimated glomerular filtration rate values and in patients requiring hemodialysis if no other relevant treatment options are available[94,119,120]. The meta-analysis included 21 studies with 717 patients in CKD stages 4-5, demonstrating an SVR of 97% and a good safety profile of SOF-containing regimens[121]. Therapy should be used with caution, and renal parameters should be carefully monitored during the treatment course due to the possibility of worsening renal function, as demonstrated by Saxena *et al*[122].

Among GT-specific options, the OBV/PTV/r +/- DSV and GZR/EBR regimens are registered for use in patients with renal failure, including those on dialysis[44,123]. The RUBY-1 and RUBY-2 clinical trials demonstrated an SVR of 95% in HCV-infected patients in CKD stage 4-5 treated with OBV/PTV/r +/- DSV +/- RBV, while the therapy with GZR/EBR in this patient population evaluated in C-SURFER study resulted in 99% efficacy[124,125]. Of the pangenotypic options, the GLE/PIB regimen has been registered for patients with severe renal failure, including those on dialysis; the good safety profile and high efficacy of this combination were documented in clinical trials[78,126].

Patients with compensated liver cirrhosis

The efficacy achieved in compensated liver patients with cirrhosis treated with GT-specific options ranged from 92% to 96%, while clinical trials evaluating pangenotypic regimens in this patient population showed an SVR of 96% and above[67,76,127,128]. Although these results corresponded with high treatment efficacy, they were lower compared to SVR in patients without cirrhosis[67,76,129]. Data from the RWE study confirmed the worse effects of therapy in the population with cirrhosis than in patients without cirrhosis. Moreover, decompensation of liver function was identified as an independent negative predictor of SVR[69,110,130]. It should be noted that IFN-based therapies were absolutely contraindicated in patients with decompensated cirrhosis due to the side effects of this cytokine[131]. Also, not all DAA regimens are registered for use in this patient population. Combinations containing protease inhibitors are not recommended because of the risk of worsening liver function[94]. Among GT-specific regimens, the SOF/LDV option is approved for use in patients with decompensated cirrhosis. Among pangenotypic regimens, it applies to SOF ± DCV ± RBV and SOF/VEL combinations[132-134].

Other factors affecting treatment outcome

Male sex is recognized as a factor that may reduce the effectiveness of DAA therapy. Multivariate logistic regression models performed in a large retrospective analysis of 17487 United States veterans with HCV infection found that the male sex was an independent negative predictor of achieving SVR [43]. These data confirm the results of other RWE analyses conducted in numerically smaller populations[110,135].

Patients with GT3 infection, who were considered an easy-to-treat population in the era of IFN-based therapy, are less likely to respond to treatment in the era of DAAs[43,136,137].

RESCUE REGIMENS

Despite the very high efficacy of currently available DAA therapy exceeding 95%, there is still a small proportion of patients who do not respond to treatment, especially when different negative predictors of achieving SVR are present[113,138]. Failure of therapy with DAA regimens could be related to the selection of mutant-resistant strains carrying resistance-associated substitutions, and those contributing

lower sensitivity to NS5A inhibitors are the most persistent[139]. The only therapeutic option registered for treatment in such cases is a three-drug pangenotypic combination of SOF/VEL/VOX. The introduction of this regimen improved the SVR rates to 96%-98% in patients after the failure of DAA therapy evaluated in POLARIS-1 and POLARIS-4 clinical trials[84,80]. The findings from registration studies are supported by data from routine medical practice[113,140,141]. A meta-analysis of 10 cohorts including 1187 patients demonstrated that 96% of them achieved undetectable HCV RNA 12 wk after the end of treatment. However, lower effectiveness was reported for those with GT3 infection and cirrhosis[140,141].

According to the most recent European Association for the Study of the Liver and American Association for the Study of Liver Diseases guidelines, in patients who have failed SOF/VEL/VOX, extended retherapy with this regimen plus weight-based RBV or a combination of GLE/PIB + SOF + RBV is recommended, although no data from clinical trials for SOF/VEL/VOX nonresponders are available[94,142]. It was pointed out that retreatment can be optimized based on resistance-associated substitution testing[94]. A 96% efficacy of the rescue regimen of GLE/PIB in combination with SOF and RBV was evaluated in the MAGELLAN-3 clinical trial, but all 23 included participants were GLE/PIB failures[143].

Very limited data on patients receiving GLE/PIB + SOF +/- RBV retherapy are available from RWE studies. In an open-label study conducted in New Zealand, patients treated with GLE/PIB + SOF after prior DAA failure achieved a virologic rate of 98%[144].

CONCLUSION

Since the beginning of the availability of IFN-free options, we have observed changes occurring in the regimens used from GT-specific to highly potent pangenotypic options, including rescue combinations. These are accompanied by changes in the profile of the HCV-infected population, with patients starting treatment over time being younger, with less advanced liver disease and less burdened by comorbidities. Despite the very high effectiveness of current DAA options, some factors including male sex, GT3 infection, liver cirrhosis and prior failure of DAA therapy are still identified that lower the chances of a cure.

FOOTNOTES

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Country/Territory of origin: Poland

ORCID number: Michał Brzdęk 0000-0002-1180-9230; Dorota Zarębska-Michaluk 0000-0003-0938-1084; Robert Flisiak 0000-0003-3394-1635.

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REVIEW

Periodontal treatment and microbiome-targeted therapy in management of periodontitis-related nonalcoholic fatty liver disease with oral and gut dysbiosis

Ryutaro Kuraji, Takahiko Shiba, Tien S Dong, Yukihiro Numabe, Yvonne L Kapila

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Ryutaro Kuraji, Department of Periodontology, The Nippon Dental University School of Life Dentistry at Tokyo, Tokyo 102-0071, Japan

Ryutaro Kuraji, Yvonne L Kapila, Department of Orofacial Sciences, University of California San Francisco, San Francisco, CA 94143, United States

Takahiko Shiba, Department of Oral Medicine, Infection, and Immunity, Harvard School of Dental Medicine, Boston, MA 02115, United States

Takahiko Shiba, Department of Periodontology, Tokyo Medical and Dental University, Tokyo 113-8549, Japan

Tien S Dong, The Vatche and Tamar Manoukian Division of Digestive Diseases, University of California Los Angeles, Department of Medicine, University of California David Geffen School of Medicine, Los Angeles, CA 90095, United States

Yukihiro Numabe, Department of Periodontology, The Nippon Dental University School of Life Dentistry at Tokyo, Tokyo 102-8159, Japan

Yvonne L Kapila, Sections of Biosystems and Function and Periodontics, Professor and Associate Dean of Research, Felix and Mildred Yip Endowed Chair in Dentistry, University of California Los Angeles, Los Angeles, CA 90095, United States

Corresponding author: Ryutaro Kuraji, DDS, PhD, Associate Professor, Lecturer, Department of Periodontology, The Nippon Dental University School of Life Dentistry at Tokyo, 1-9-20, Fujimi, Chiyoda-ku, Tokyo 102-0071, Japan. r-kuraji@tky.ndu.ac.jp

Abstract

A growing body of evidence from multiple areas proposes that periodontal disease, accompanied by oral inflammation and pathological changes in the microbiome, induces gut dysbiosis and is involved in the pathogenesis of nonalcoholic fatty liver disease (NAFLD). A subgroup of NAFLD patients have a severely progressive form, namely nonalcoholic steatohepatitis (NASH), which is characterized by histological findings that include inflammatory cell infiltration and fibrosis. NASH has a high risk of further progression to cirrhosis and hepato-cellular carcinoma. The oral microbiota may serve as an endogenous reservoir for gut microbiota, and transport of oral bacteria through the gastro-intestinal tract

can set up a gut microbiome dysbiosis. Gut dysbiosis increases the production of potential hepatotoxins, including lipopolysaccharide, ethanol, and other volatile organic compounds such as acetone, phenol and cyclopentane. Moreover, gut dysbiosis increases intestinal permeability by disrupting tight junctions in the intestinal wall, leading to enhanced translocation of these hepatotoxins and enteric bacteria into the liver through the portal circulation. In particular, many animal studies support that oral administration of *Porphyromonas gingivalis*, a typical periodontopathic bacterium, induces disturbances in glycolipid metabolism and inflammation in the liver with gut dysbiosis. NAFLD, also known as the hepatic phenotype of metabolic syndrome, is strongly associated with metabolic complications, such as obesity and diabetes. Periodontal disease also has a bidirectional relationship with metabolic syndrome, and both diseases may induce oral and gut microbiome dysbiosis with insulin resistance and systemic chronic inflammation cooperatively. In this review, we will describe the link between periodontal disease and NAFLD with a focus on basic, epidemiological, and clinical studies, and discuss potential mechanisms linking the two diseases and possible therapeutic approaches focused on the microbiome. In conclusion, it is presumed that the pathogenesis of NAFLD involves a complex crosstalk between periodontal disease, gut microbiota, and metabolic syndrome. Thus, the conventional periodontal treatment and novel microbiome-targeted therapies that include probiotics, prebiotics and bacteriocins would hold great promise for preventing the onset and progression of NAFLD and subsequent complications in patients with periodontal disease.

Key Words: Periodontal disease; Nonalcoholic fatty liver disease; Microbiota; Dysbiosis; Metabolic syndrome; Probiotics

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Core Tip: A growing body of evidence from multiple areas highlights that periodontal disease, accompanied by oral inflammation and pathological changes in the microbiome, induces gut dysbiosis and is involved in the pathogenesis of Nonalcoholic fatty liver disease (NAFLD). Thus, the conventional periodontal treatment and microbiome-targeted therapies that include probiotics, prebiotics and bacteriocin would hold great promise for preventing the onset and progression of NAFLD. In this review, we will describe the link between periodontal disease and NAFLD with a focus on basic, epidemiological, and clinical studies, and discuss potential mechanisms linking the two diseases and possible therapeutic approaches focused on the microbiome.

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INTRODUCTION

Periodontal disease is an inflammatory disease that is induced by a structural and metabolic imbalance of the oral microbiome, namely an oral dysbiosis, and is characterized by significant periodontal tissue destruction and tooth loss[1-3]. Until recently, specific periodontopathic bacteria, such as the Red Complex, were thought to be the major pathogens of periodontal disease[4,5], but the success of novel methods focused on the studying the oral microbiome with next-generation sequencing approaches has revealed the possibility that a variety of previously unknown bacteria, fungi, and even viruses are associated with the periodontal disease[6-9].

Periodontal disease has also been reported to adversely affect the pathogenesis of various systemic diseases, including diabetes, coronary vascular disease, rheumatoid arthritis, and cancer[10,11]. As a major mechanism, it has been proposed that pro-inflammatory cytokines, periodontal pathogens, and their microbial components spread out from damaged periodontal tissues sites into the systemic circulation and thereby reach and affect other organ sites[1,12-14]. Another idea has also emerged in the last decade that there is enteric translocation following the development of gut dysbiosis that originates from oral bacteria, and this is another pathway linking periodontitis and systemic disease[11]. Many studies have shown that swallowed periodontopathic bacteria can pass through the gastric acid barrier and reach the gut, thereby shifting the gut microbiome to an unhealthy state[15-19]. In addition, even oral commensal bacteria that are not normally pathogenic may proliferate and become established in the

gut environment due to periodontal inflammation, and may even manifest pathogenic properties[20,21].

In the context of the association between periodontal disease and gut dysbiosis, nonalcoholic fatty liver disease (NAFLD) has received particular attention in recent years[11,22] (Figure 1). NAFLD is currently the most prevalent chronic liver disease worldwide, with a marked increase in individuals with metabolic syndrome[23], and it is defined as a fatty liver condition diagnosed upon histological observation or image analysis, but other liver diseases/conditions, such as a history of alcohol consumption, viral hepatitis, and drug-induced liver injury are excluded[24,25]. The majority of NAFLD patients (approximately 80%) have a good prognosis and simply have a fatty liver (NAFL), whereas the remaining 10%-20% have a severely progressive form, namely nonalcoholic steatohepatitis (NASH)[25]. In addition to lipid deposition, NASH is characterized by histological findings that include inflammatory cell infiltration, ballooning degeneration and fibrosis of hepatocytes, and a reported high risk of further progression to end-stage liver disease, such as cirrhosis and hepatocellular carcinoma[26,27]. NAFLD/NASH is considered a liver phenotype within various metabolic syndrome components and has a robust and bidirectional association with metabolic complications such as diabetes, obesity, and cardiovascular disease[28].

Several animal studies have demonstrated that periodontopathic bacteria, such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* cause increased insulin resistance and glucose intolerance through gut dysbiosis, as well as liver inflammation and fat deposition and fibrosis[29-33]. These relationships between periodontal bacteria and NAFLD are also supported by epidemiological studies[30,34-37], including cross-sectional and cohort studies, and further strengthened by clinical studies reporting that periodontal treatment improved NAFLD[36,38].

The liver is in a unique anatomic location and as such it has a dual blood supply. The liver gets blood from the systemic circulation and the gut, with the portal vein responsible for most of this blood supply, and it transports nutrients, bacterial metabolites, toxins, and drugs absorbed from the gut to liver[39,40]. In gut dysbiosis, abnormal growth and structural changes in intestinal bacteria result in an increase in hepatotoxic substances, such as endotoxin and ethanol, which reach the liver *via* the portal circulation [41-44]. In addition, increased intestinal permeability due to gut dysbiosis accelerates these hepatic exposures[45,46].

Thus, management of the oral and gut microbiome may be an important preventive strategy for patients with periodontal disease and NAFLD (Figure 2). In this regard, besides traditional therapies, the use of adjunctive therapies that include probiotics and prebiotics may be beneficial in normalizing dysbiotic microbiomes present in periodontal tissues, the liver, and the gut. This review will describe the link between periodontal disease and NAFLD with a focus on basic, epidemiological, and clinical studies. Potential mechanisms linking the two diseases and possible therapeutic approaches focused on the microbiome will also be discussed.

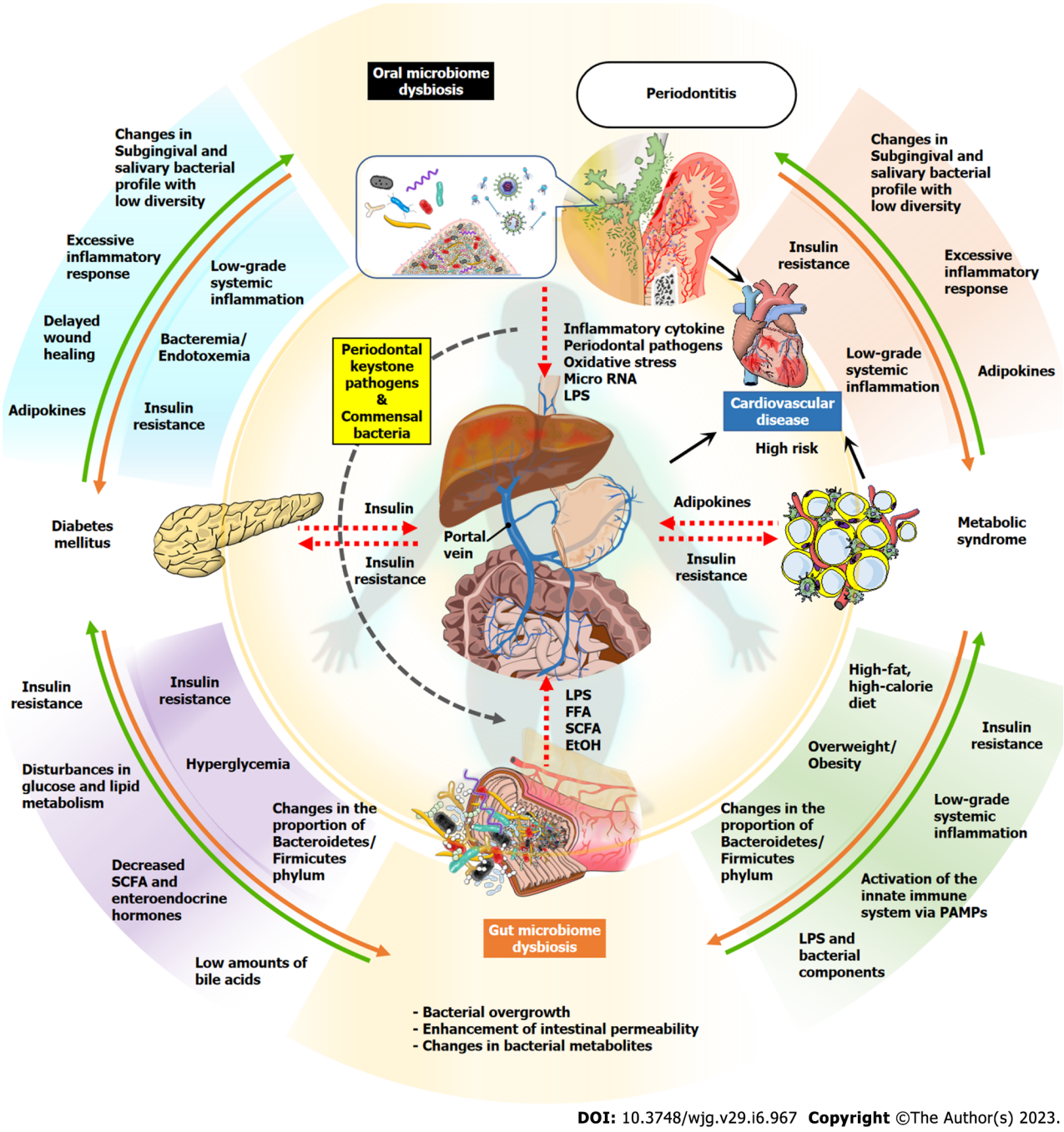
PERIODONTAL DISEASE AS A RISK FACTOR FOR SYSTEMIC DISEASES

Periodontitis, a chronic inflammatory disease is characterized by a microbial dysbiosis and a progressive destruction of the tooth supporting structures[47]. In the United States, Periodontitis affects 42.2% of the population over the age of 30 and 59.8% over the age of 65[48]. Periodontitis is the major cause of tooth loss in adults, according to data from the World Health Organization[49]. Periodontitis has a multifactorial pathogenesis that includes environmental, microbial, and host factors which affect disease outcomes. Several systemic diseases and conditions have been associated with periodontal disease, including cardiovascular disease, diabetes mellitus, and metabolic syndrome[11,50-54].

The impact of systemic diseases and disorders on the periodontal tissues is well known, and the evidence indicates that periodontal disease may significantly enhance the risk for certain systemic diseases or conditions or alter their natural course[55-60]. Many systemic conditions have been associated with periodontal diseases, although the level of evidence for each condition varies. Conditions for which the influence of periodontal disease is well documented include coronary heart disease and related events, diabetes mellitus, preterm and low-birth-weight delivery, and preeclampsia; and respiratory conditions, such as chronic obstructive pulmonary disease[55-57]. A smaller but growing evidence base supports an association between poor oral health, tooth loss, or periodontitis and conditions, such as chronic kidney disease and renal insufficiency[61-65]; certain forms of cancer[66-70] affecting the liver, pancreas, and colorectal region; rheumatoid arthritis[71,72] and altered cognitive function, dementia, and Alzheimer disease[22,55,73-77]. Here we will discuss the influence of periodontal disease in the context of diabetes and metabolic syndrome (Figure 1).

Diabetes

In terms of diabetes, a variety of studies have examined the effects of diabetes on the periodontal tissues, whereas others have examined the effect of periodontal disease on the control of diabetes[56]. A review of several studies including those with more than 22000 patients found that the incidence of type 2 diabetes (*i.e.*, new diagnoses) was significantly greater in individuals with periodontal disease than in those without periodontal disease[78]. Severe periodontal disease was associated with a significant

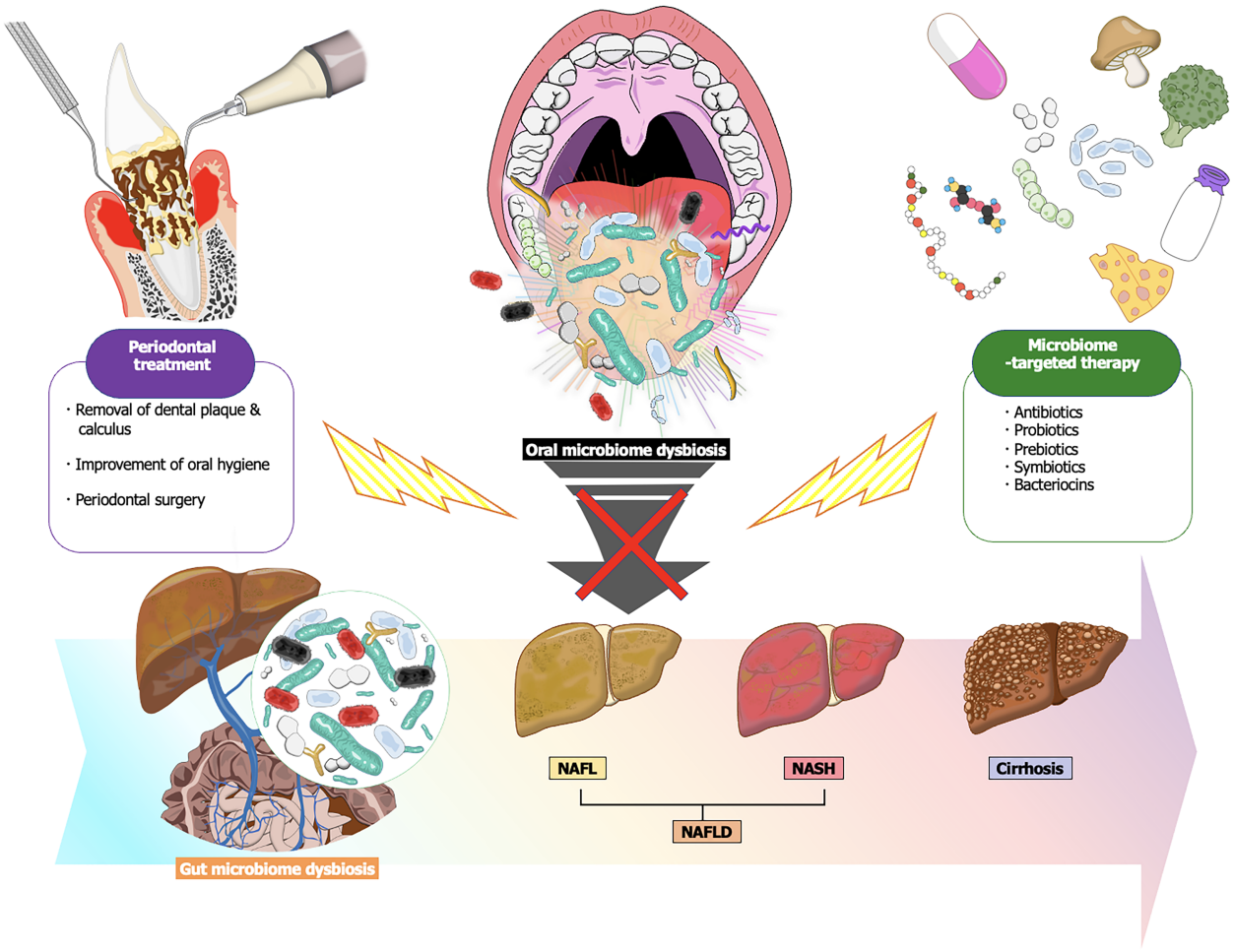


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Figure 1 Circular model of crosstalk between periodontal disease, gut microbiota, diabetes mellitus, and metabolic syndrome in the pathogenesis of nonalcoholic fatty liver disease. LPS: Lipopolysaccharide; FFA: Free fatty acids; PAMPs: Pathogen-associated molecular pattern molecules; SCFA: Short-chain fatty acids; EtOH, Ethanol.

worsening of glycemic control over time in patients with type 2 diabetes[79]. Those with severe periodontitis at baseline had a greater incidence of worsening glycemic control over a 2- to 4-year period as compared with those without periodontitis at baseline. Furthermore, periodontitis was known to have preceded the worsening of glycemic control in this study. In addition, numerous systematic reviews and meta-analyses have consistently shown that periodontal therapy is associated with a significant and clinical improvement in glycemic control in patients with diabetes and periodontitis[80-82]. Furthermore, meta-analyses also indicate a higher prevalence and severity of periodontal disease in diabetic patients and vice versa[83,84].

Understanding the mechanisms that underlie other infections is helpful to understanding the mechanisms whereby periodontitis influences glycemic control. Systemic inflammation is well known to play a major role in insulin sensitivity and glucose dynamics. Periodontitis can trigger or sustain an elevated systemic chronic inflammatory state, as shown by increased levels of serum C-reactive protein (CRP), interleukin (IL)-6, and fibrinogen in individuals with periodontal disease[79,85,86]. Inflammation triggers insulin resistance, which often accompanies systemic infections. For example, acute viral and



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Figure 2 Periodontal treatment and microbiome-targeted therapy in nonalcoholic fatty liver disease patients with periodontitis based on the oral-gut-liver axis concept. NAFL: Nonalcoholic fatty liver; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis

bacterial infections can increase insulin resistance and aggravate glycemic control[87,88]. Systemic infections increase insulin resistance through several mechanisms. The insulin resistance, in turn, prevents glucose from entering target cells, causing elevated blood glucose levels. The elevated blood glucose then requires increased pancreatic insulin production to maintain normal glycemic levels. The insulin resistance can persist for weeks or months after the individual has recovered from the illness. Individuals with type 2 diabetes who already have significant insulin resistance, may experience additional tissue resistance to insulin as a result of infection, and thus exhibit an exacerbated poor glycemic control. In addition, studies suggest a relationship between the inflammatory status associated with diabetes or metabolic syndrome and the inflammatory status of periodontal disease[89,90]. Indeed chronic tissue inflammation has been recognized in the onset of metabolic diseases[91].

Chronic gram-negative periodontal infections may also lead to increased insulin resistance and poor glycemic control[92]. In individuals with periodontal disease, persistent systemic exposure to periodontopathic bacteria and their products results in an up-regulation of the immunoinflammatory response, with elevation in serum levels of proinflammatory mediators, such as IL-1β, tumor necrosis factor alpha (TNF-α), and IL-6, similar to well-recognized systemic infections, but on a more persistent, chronic basis. Increased serum levels of several cytokines, including TNF-α and IL-6, are associated with increased insulin resistance. These processes would help explain the worsening glycemic control associated with severe periodontal disease. Periodontal treatment, which is meant to decrease the bacterial insult and reduce inflammation, may lead to decreased systemic inflammation that may restore insulin sensitivity over time, and thereby result in improved metabolic control. This hypothesis would be supported by the many studies showings the improved glycemic control following periodontal therapy.

Much focus has been placed on the fact that chronic conditions can change the microbiome. As an example, individuals exhibiting obesity or type 2 diabetes demonstrate a modified gastrointestinal microbiome (reviewed by[93-96]). In addition, rodents exposed to *Enterobacter cloacae* B29, an intestinal microbe obtained from obese subjects with diabetes, develop insulin resistance and obesity[97]; indicating that bacteria can also directly induce diabetes-related symptoms. Also, modulating the microbial-mediated mucosal immunity and inflammation may help attenuate type 2 diabetes; by

mechanisms that include modulation of T lymphocytes[98,99]. Since there are associations between type 2 diabetes, periodontal disease, and changes in the gut microbiome of individuals with type 2 diabetes, additional focus has been placed on microbiome changes in the periodontal tissues of individuals with type 2 diabetes.

Studies have shown that type 2 diabetes alters the subgingival and salivary microbial profile by lowering richness and diversity[100-105]. These findings support the observations found in the gut microbiome[93-96,106]. Studies show that the subgingival microbiome diversity is not only decreased in individuals with type 2 diabetes, but when separated by adequate or inadequate glycemic control, there is a further decrease in the microbiome diversity in those with inadequate glycemic control (hemoglobin A1c 8)[107]. However, another study which assessed the microbiome present in the saliva of 17 subjects with periodontal disease and type 2 diabetes was not able to find a relationship between glycemic control and changes in the oral microbiota. Although, the study found that in individuals with type 2 diabetes, the microbial composition varied significantly between obese (body mass index = 30) and non-obese individuals with type 2 diabetes, such that obesity reduced the oral microbial diversity[108]. Furthermore, although the subgingival and supragingival microbial diversity decreases when subjects with type 2 diabetes are compared to normoglycemic individuals, the microbial shift is less in individuals with periodontal disease with type 2 diabetes subjects than in normoglycemic individuals [101,102,109].

In summary, the subgingival and salivary microbial profile is altered by type 2 diabetes by decreasing microbial diversity and richness (Figure 1). In terms of glycemic control, there is a further decrease in microbial diversity when there is inadequate glycemic control. Furthermore, the microbial shift observed in individuals with periodontal disease is less prominent in individuals with type 2 diabetes compared to normoglycemic controls. In addition, microbial diversity is further reduced in smokers. Additional research is needed to examine a potential two-way relationship between diabetes and the periodontal microbiome; namely that periodontal microbes may directly induce diabetes-related pathology, as data indicate that microbes derived from the gastrointestinal tract of obese patients with diabetes can themselves mediate symptoms related to diabetes in animal models.

Metabolic syndrome

We will next discuss the association between metabolic syndrome and periodontal disease. Metabolic syndrome is a characterized by constellation of conditions that elevate the risk for cardiovascular disease and double the risk for type 2 diabetes[110-113]. Approximately 34% of the US population[114] and 10% of US adolescents are affected by Metabolic syndrome[115]. Metabolic syndrome prevalence increases with age and varies with ethnicity and gender[116]. Metabolic syndrome is defined slightly differently by different agencies. That conveyed by the National Cholesterol Education Program Adult Treatment Panel III is the most frequently used definition; an individual has to exhibit at a minimum, three out of the five risk factors: (1) Low plasma levels of high density lipoprotein cholesterol; (2) Increased values for plasma triglycerides; (3) Increased abdominal circumference; (4) Elevated blood pressure; and (5) Elevated glucose levels[117]. Pre-diabetes is included in metabolic syndrome as it presents with insulin resistance and is highly predictive of new-onset type 2 diabetes[118].

Abdominal obesity and insulin resistance are primary risk factors for metabolic syndrome are. Physical inactivity, aging, and hormonal imbalance also contribute to metabolic syndrome[119]. A primary trigger and risk factor for the majority of mechanisms in metabolic syndrome is visceral adiposity[120]. Precise processes that underlie the systemic response seen in metabolic syndrome have not been well elucidated, but data highlight that the inflammatory response present this disease mediates endothelial dysfunction that may, in turn, promote type 2 diabetes and cardiovascular disease [121-123].

There has been a significant interest in the association between metabolic syndrome and periodontal disease, since both are characterized by insulin resistance and systemic inflammation[124,125]; common pathways whereby they could impact each other. Cross-sectional, longitudinal, and meta-analyses studies have evaluated the association between metabolic syndrome and periodontal disease. Most data indicates that there is an association between metabolic syndrome and periodontal disease[126-130]. Three meta-analyses, discussed here, found an association between periodontal disease and metabolic syndrome (odds ratio from 1.38 to 1.99)[131-133]. One meta-analysis[133] that discussed 39 studies showed an association between metabolic syndrome and periodontal disease [crude odds ratio of 1.99 (95%CI: 1.75-2.25) and an adjusted odds ratio of 1.46 (95%CI: 1.31-1.61)]. A subgroup analysis focused on different countries was also included in this study and the results showed a combined odds ratio of 1.75 (95%CI: 1.31-2.34) for the United States; 1.68 (95%CI: 1.41-2) for Japan; 1.81 (95%CI: 1.35-2.42) for Korea, and 2.29 (95%CI: 1.53-3.41) for China[133]. Another meta-analysis[131] of 26 manuscripts that included radiographic and clinical exam data reported an association between these two diseases [odds ratio of 1.38 (95%CI: 1.26-1.51)]. This suggested that patients with metabolic syndrome are 38% more likely to exhibit periodontal disease[131]. A systematic review/meta-analysis of multiple manuscripts that reported on 36,337 individuals, found a positive association between periodontal disease and metabolic syndrome [odds ratio of 1.71 (95%CI: 1.42-2.03)][132]. A comprehensive review further found a positive association between metabolic syndrome and periodontal disease[134]. Furthermore, various animal studies that employed different periodontal disease models demonstrated that rodents with

metabolic syndrome or obesity due to a high-fat diet, also exhibited exacerbated periodontal bone loss [135-139].

In terms of assessing metabolic syndrome's role in contributing to periodontal disease, two longitudinal studies found that metabolic syndrome increases the risk of periodontal disease development and progression[140,141]. Specifically, one study found that individuals with metabolic syndrome were 2.6 times more likely to develop periodontal disease. Furthermore, with increased attributes of metabolic syndrome, the periodontal disease exhibits a higher prevalence and a more extensive presentation[141,142]. Even though most studies found an association between periodontal disease and metabolic syndrome, some reports found a minimal association for these diseases[143-148]. However, most of the studies that found no association were either cross-sectional or shorter longitudinal studies (only one year), and one study was performed on a young cohort of subjects[147], that had low levels of both metabolic syndrome and periodontal disease. Furthermore, in longitudinal study of three years, researchers found that toothbrushing frequency was inversely related to the incidence of metabolic syndrome[149].

Most studies have concluded that periodontal disease may contribute to the development or exacerbation of metabolic syndrome[150-152]. In a cross-sectional study of 190 individuals, researchers found that periodontal disease and periodontal bone loss may contribute to the development of metabolic syndrome[150]. A four-year longitudinal study of 1023 adults found deeper periodontal pockets associated with a positive conversion of metabolic components (odds ratio 1.6; 95%CI: 1.1-2.2)[151]. Furthermore, another study reported that in individuals with metabolic syndrome a decrease in periodontal inflammation reduced C-reactive protein levels in those subjects[152].

There has been an increasing interest in the role of the microbiome in metabolic diseases and disturbances. Metabolic diseases change the gut microbiome (reviewed by[96]), and the oral microbiome varies significantly between individuals with a healthy periodontium and those with periodontal disease[153]. Furthermore, changes in the gastrointestinal microbiome are associated with metabolic syndrome and obesity[154]. Obesity affects the oral microbiome in the context of type 2 diabetes[108], plus it lowers the diversity of the microbiome within the distal gut[155,156]. Further, subjects with decreased microbial diversity exhibit significantly more insulin resistance, adiposity, and dyslipidemia in contrast to individuals with elevated microbial richness[156]. A study focused on 17 subjects with type 2 diabetes and with advanced periodontal disease found that the composition of the oral microbiome varied significantly between non-obese and obese (BMI = 30) subjects. Those findings suggested that obesity is related to a decreased oral microbial diversity[108]. Our research group also showed that the oral microbiome is altered in individuals with metabolic syndrome as compared to healthy individuals[157]. In addition, there are a growing number of studies suggesting potential links between the gut or oral microbiome and obesity[158-160].

In vitro and *in vivo* (animal) studies have shown how dyslipidemia (high-fat diet) compounds the effects of metabolic syndrome on periodontitis[135-139,161], and a variety of studies have assessed the role of impaired glucose in periodontal disease. A study on obese mice (metabolic syndrome model) inoculated with *P. gingivalis* and fed a high-fat diet that did not exhibit diabetes, revealed that these mice had 40% more alveolar bone loss and higher levels of *P. gingivalis* versus control mice[135]. Using the same model, another group of researchers found that the high-fat diet group exhibited metabolic syndrome, including dyslipidemia, obesity, hyperinsulinemia, and insulin-resistance. Further, the group with metabolic syndrome exhibited significantly higher osteoclast formation and alveolar bone loss. Similarly, our previous study showed that the ligature-induced periodontitis with *P. gingivalis* infection in rats fed a high-fat diet not only exacerbated the alveolar bone resorption, but also induced increase in levels of fasting blood glucose and liver damage markers, and systemic inflammation[138,139].

Moreover, lipopolysaccharide (LPS) induced-periodontitis exacerbated inflammatory cytokine expression (IL-6, monocyte chemoattractant protein-1, receptor activator of NF-kappaB ligand, and macrophage colony-stimulating factor), osteoclast formation, and alveolar bone loss[137]. Studies that used osteoblasts from obese mice showed a reduction in cell proliferation and an elevated osteoblast cell death following *P. gingivalis* treatment *vs* controls[162]. Furthermore, obese rodents with metabolic syndrome exhibited significant elevated levels of *A.actinomycetemcomitans*-LPS-induced alveolar bone loss versus the non-obese, non-metabolic syndrome animals. In addition, a cholesterol-lowering medication frequently given to patients with metabolic syndrome[163], attenuated alveolar bone loss in both groups, further supporting the role of dyslipidemia in periodontal inflammation[164]. In this regard, our research found that submucosal administration of radio-labeled *P. gingivalis* LPS to the palatal gingiva of high-fat diet-induced obese rats led to a significant accumulation of LPS in the liver and gingiva compared to other organs, including the spleen, kidney, and brain. Furthermore, the gingiva and fatty liver of obese rats demonstrated an increased level of *P. gingivalis* LPS and a prolonged accumulation time compared to mice fed a normal diet. In addition, a high-fat diet further delayed the metabolic clearance of *P. gingivalis* LPS from the gingiva and liver[165].

The role of lipids in periodontal disease was further explored using *in vitro studies*, and these studies showed that fatty acids augmented the LPS-mediated expression of markers involved in periodontal disease, for example IL1-a, IL1-b, C-X-C motif chemokine 10, cluster of differentiation (CD) 86, colony stimulating factor 2, monocyte chemoattractant protein-1, toll-like receptor 2, TNF-α, and CD14[137]. Further *in vitro* studies performed on macrophages demonstrated a significant increase in CD36, a major

fatty acid receptor, upon treatment with LPS plus palmitate in comparison to macrophages only treated with LPS or palmitate alone[161]. Periodontal disease and metabolic syndrome, independently and significantly increased the levels of CD36, and when metabolic syndrome and periodontal disease were present simultaneously, there was an additive effect. The expression of CD36 in periodontal tissues also positively correlated with osteoclast formation.

Taken in aggregate, human studies and animal models show an association between metabolic syndrome and periodontal disease, and metabolic syndrome can change the oral microbiome and potentiate the harmful effects of periodontal disease.

RELATIONSHIP BETWEEN PERIODONTAL DISEASE AND NAFLD

Periodontal disease has been associated with liver diseases, such as NAFLD, liver cirrhosis, and hepatocellular carcinoma, and even a possible worse prognosis for liver transplantation (Figure 1). The relationship between periodontitis and NAFLD is supported by a number of epidemiological studies[30,34-36,166-170]. Cross-sectional studies have also shown that the severity of periodontal disease correlates significantly with levels of blood liver injury markers [*e.g.* alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transpeptidase[171-173], hepatic imaging by ultrasound and computerized tomography, and a formula-based scoring system for estimating the degree of fatty liver and liver fibrosis[166,174,175]. Furthermore, several cohort studies have strengthened the causal relationship between periodontal disease and NAFLD. Akinkugbe *et al*[176] followed 2623 non-NAFLD subjects for more than 5 years (mean 7.7 years) in a health survey conducted in West Pomerania in Germany, and found that a history of periodontitis was an independent risk factor contributing to the development of NAFLD, which was diagnosed by abdominal ultrasonography and serum ALT levels. Compared to subjects without clinical attachment level (CAL) ≥ 3 mm, the incidence of NAFLD was significantly higher in patients with < 30% or ≥ 30% affected sites with CAL ≥ 3 mm. The adjusted incidence rate ratio for NAFLD was 1.28 for < 30% and 1.60 for ≥ 30% of affected sites with a statistically significant difference, respectively. Similarly, the incidence rate difference was statistically significant at 5.49 for < 30% of sites and 11.11 for ≥ 30% of sites. Helenius-Hietala *et al*[34] found in a 13-year cohort study of 6165 subjects participating in a Finnish population-based health study, the proportion of sites with deep periodontal pockets positively correlated with the hazard ratio for developing severe hepatic diseases. In addition, the hazard ratio was not correlated with periodontitis in those who were non-NAFLD at the study entry, whereas was 6.94 in those who were developed NAFLD with significant correlation. Based on systematic reviews and meta-analyses, this accumulating evidence highlights the potential for periodontal disease as a direct and indirect risk factor for the development and progression of NAFLD.

Periodontal disease has been reported to exacerbate metabolic diseases including diabetes, dyslipidemia and obesity through disturbance of energy homeostasis, and given the close association between NAFLD and metabolic syndrome, this underscores the importance of understanding the periodontal-metabolic relationship in the NAFLD pathogenesis. Two-thirds of patients with obesity and type 2 diabetes have been found to have fatty liver, and both excessive body mass index and visceral obesity are recognized as risk factors for NAFLD[177,178]. Metabolic syndrome is also related to an increased risk of more progressive liver disease, such as NASH, cirrhosis, and future liver failure, in patients with NAFLD[179-181]. A 5 year-follow up cohort study reported by Kuroe *et al*[182] demonstrated that in 341 Japanese subjects with NAFL without complications of liver fibrosis, stratified analysis by obesity showed that in obese subjects, severity of periodontal destruction was significantly correlated with liver fibrosis with an odds ratio of 2.87, whereas in non-obese subjects they were not correlated. Moreover, in metabolic diseases animal models induced by high-fat diet or high-cholesterol diet, the ligature-induced experimental periodontitis and periodontopathic bacterial infection exacerbated disturbances of glycolipid-metabolism in the liver and enhance the NAFLD progression[138,183,184]. Although not all mechanisms explaining the interaction between periodontal disease and NAFLD *via* metabolic diseases are known, diffusion of inflammatory mediators and periodontopathic bacteria and their components, and release of reactive oxygen species from inflamed periodontal tissues into the circulation can mediate low-glade systemic inflammation and, thereby exacerbate insulin resistance in obesity and diabetes [185].

Data over the last 10 years strongly suggest that *P. gingivalis* is involved in NAFLD and NASH. *P. gingivalis* has many virulence factors, such as collagenase, trypsin-like gingipain enzymes, LPS and fimbriae, that are also known to trigger intracellular signaling events[186]. Yoneda *et al*[36] analyzed various periodontopathic bacteria in saliva collected from non-NAFLD control subjects and NAFLD patients using polymerase chain reaction assays and showed that the detection frequency of *P. gingivalis* was significantly higher in the NAFLD patients. In addition, injection of *P. gingivalis via* the jugular vein dramatically accelerated NAFLD progression in mice fed a high fat diet. We have previously reported that *P. gingivalis* infection combined with ligature-induced periodontitis increased serum ALT and LPS levels and hepatic fat deposition in high-fat diet-induced obese rats with insulin resistance[138,139]. Conversely, hyperglycemia can promote translocation of *P. gingivalis* from the oral cavity to the liver

and reduce hepatic insulin-induced glycogen biosynthesis in mice[187]. For other periodontopathic bacteria, enteral infection with *Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia* as well as *P.gingivalis* may contribute to NAFLD by altering the gut bacterial flora and metabolome in mice[30,32].

MECHANISMS BY WHICH PERIODONTAL DISEASE EXACERBATES NAFLD BASED ON THE ORAL-GUT-LIVER AXIS

At the present time, dual pathways have been proposed to explain the connection between periodontal disease and liver pathology, namely hematogenous and enteral diffusion of hepatotoxic components [11]. The liver is the largest parenchymal organ and, due to its unique anatomical location, it has a dual blood supply coming from the systemic circulation and the gastrointestinal tract[39], which helps explain how toxic factors diffuse from periodontal tissue and reach the liver. Eighty % of the total blood supply received by the liver is derived from the enteric portal vein, which is rich in nutrients, bacterial metabolites, and food antigens. The remaining 20% is supplied by the hepatic artery, which is the nutrient vessel of the liver and branches off the abdominal aorta. Thus, the liver is the hemodynamic confluence in the human body, and its unique characteristics allow for the maintenance of a diverse intrahepatic cell population composed of metabolically active hepatic parenchymal cells, connective tissue cells, and various immune cells[40].

One of the pathways linking the oral cavity and liver is thought to be hematogenous diffusion, which is caused by inflammatory mediators and microorganisms passing through the systemic circulation due to hyperpermeability of the pocket epithelium and micro-ulceration in the periodontal inflamed tissue [1,12]. For example, the serum levels of reactive oxygen species and inflammatory cytokines are elevated in periodontitis patients, suggesting a mild chronic inflammatory state systemically[188,189]. In fact, epidemiological studies have shown that Elevated blood level of CRP, which is secreted by hepatocytes upon stimulation of proinflammatory cytokines, are a common modifier of periodontitis and NAFLD [167,190]. With regard to microbiological invasion, mechanical injuries such as daily oral hygiene activities, chewing movements, and calculus removal as periodontal treatment have been reported to increase the frequency of bacteremia and endotoxemia in patients with periodontal disease compared to healthy subjects[191,192].

Another possible pathway linking the oral cavity to the liver is the transport of oral bacteria through the gastrointestinal tract, resulting in abnormalities in the gut microbiota[42,193,194]. Patients with periodontitis have a characteristic oral microbiome, and they continuously and unknowingly swallow pathogens present in saliva and dental plaque[19]. A part of oral bacteria can reach the intestinal tract, even in systemically healthy individuals and regardless of the harsh acidic environment in the stomach. Thus, the oral microbiome may function as an endogenous reservoir that supply novel bacteria to the gut microbiome[195]. It is widely known that gut dysbiosis is closely associated with the pathogenesis of NAFLD through changes in gut bacterial composition and metabolites[193,196,197]. In a state of gut dysbiosis, the production of choline which is an essential substance for lipolysis is decreased, whereas LPS, ethanol and volatile organic compounds (*e.g.* acetone, phenol and cyclopentane) are increased, which are potential hepatotoxin[41,42,44]. Moreover, the gut dysbiosis enhanced intestinal permeability by disrupting intercellular junctions in the intestinal mucosa, leading to increased translocation of enteric bacteria and their metabolites to the liver. Therefore, the liver is constantly exposed to various substances of intestinal derivation, which diffuse into the liver through the enterohepatic portal circulation.

A study by Lourenço *et al*[19] reported that numerous oral taxa related to periodontal inflammation and destruction were detected in the gut microbiome of individuals, regardless of periodontal condition. Patients with periodontal disease were characterized by a higher ratio of Firmicutes/Bacteroidetes, an enrichment in Euryarcheota, Verrucomicrobiota, and Proteobacteria, and a less diverse gut microbiota than those with healthy periodontal status. Kawamoto *et al*[16] found that fecal samples from patients with severe periodontitis were enriched in *Acidaminococcus, Clostridium, Lactobacillus, Bifidobacterium, Megasphaera*, and *Romboutsia*compared to those from healthy subjects. However, few studies have observed changes in the gut microbiota in patients with periodontal disease, and consistent trends in the gut dysbiosis remain unclear, with wide variations. In the future, it would be interesting to compare the modifications of the gut microbiota in the state of periodontal disease with those observed in NAFLD, diabetes, and obesity to elucidate the complex relationship between periodontal disease and systemic diseases.

Many animal studies have shown that orally administration of periodontal pathogens caused insulin resistance and hepatic fat deposition in rat and mice, accompanied by alterations in gut microbiota and glycolipid-metabolism[18,29-32]. In particular, *P. gingivalis,* known as a keystone pathogen, causes mucosal inflammation *via* contributing to oral and gut microbiome dysbiosis[29,198]. Yamazaki *et al*[32] showed that in an NAFLD mouse model fed a high-fat diet, oral administration of *P. gingivalis* or *P. intermedia* changed the composition of gut microbiota and blood metabolome, resulting in a shift in liver transcriptome expression toward the NAFLD pathogenic form. In contrast, application of commensal oral bacteria *Actinomyces naeslundii* and *Veillonella rogosae* did not affect NAFLD progression. Komazaki

et al[31] reported that oral infection with *A.actinomycetemcomitans* may contribute to NAFLD by altering the bacterial flora and glucose metabolism in mice. This result is also consistent with the fact that Anti-*A. actinomycetemcomitans* antibodies were positively correlated with visceral fat area, insulin resistance, and serum AST level in 52 Japanese NAFLD patients.

Recent studies have revealed that inflammation of periodontal tissue alters gut bacteria and metabolites even without specific periodontopathic bacterial infections[21,199,200]. Even commensal oral bacteria, which are not notably pathogenic in the oral cavity, such as *Enterobacter spp.* and *Klebsiella spp*. when stimulated to proliferate by ligature-induced periodontitis, can mediate ectopic intestinal colonization and may play an important role in the exacerbation of enteritis[21]. Therefore, future studies on the pathogenesis of NAFLD should not only focus on individual periodontal pathogens, but also comprehensively evaluate the relationship between changes in the oral and gut microbiota as a whole.

PERIODONTAL DISEASE FROM THE VIEWPOINT OF ORAL DYSBIOSIS

Polymicrobial diseases represent clinical and pathological conditions caused by 2 or more microorganisms[201]. The bacterial microbiome in the oral cavity is composed of more than 700 bacterial species [202] and is associated with the development and progression of oral diseases[203]. Periodontal diseases are representative oral polymicrobial diseases that involve a microbiome imbalance known as dysbiosis [204,205], leading to dysregulated host-microbial crosstalk that induces periodontal inflammation and alveolar bone loss[206]. The 2009–2010 and 2011–2012 cycles of the National Health and Nutrition Examination Survey reported that almost 50% of adults aged ≥ 30 years in the United States were affected by periodontitis[207]. In addition, periodontitis is associated with systemic diseases, such as type 2 diabetes, obesity, cardiovascular disease, preterm low birth weight, and NAFLD[30,208-212]. Therefore, a complete understanding of periodontal disease is essential to prevent a decline in quality of life.

The current theory suggests that gingival lesions are precursors of periodontitis[213]. Loe *et al*[214] demonstrated that the accumulation of bacterial plaque causes gingivitis in humans, and gingival inflammation is resolved by the removal of the plaque[235]. In addition, continued plaque accumulation in animal models induced gingivitis and gradually developed into periodontitis[215]. These results clearly indicate that bacterial plaque is involved in periodontitis. This belief was called the "nonspecific plaque hypothesis" which states that a sufficient accumulation of microorganisms results in destructive inflammation in periodontal tissues[216]. The contradiction between the nonspecific plaque hypothesis and actual clinical findings led scientists to propose the "specific plaque hypothesis". This hypothesis was that specific bacterial species were involved in the etiology of periodontitis[217]. However, potential periodontal pathogens could not be identified because approximately 30–100 bacterial species inhabit the periodontal pocket, and plenty of other bacterial species may have been unculturable[218].

Although culture methods have traditionally been useful for the identification of causative bacteria, DNA-based bacterial detection methods using molecular biology techniques have also contributed to the characterization of oral bacterial composition. Socransky *et al*[4] reported on findings relating to the specific plaque hypothesis where they targeted 40 subgingival bacterial species using the checkerboard DNA-DNA hybridization method. These bacteria were mainly divided into five groups: Red, orange, green, yellow, and purple complexes. *P. gingivalis*, *Tannerella forsythia*, and *Treponema denticola* were defined as periodontal pathogens and classified as the red complex because they exhibited a strong relationship with probing pocket depth and bleeding on probing. The orange complex was also associated with probing pocket depth and appeared to be closely related to the red complex. Red and orange complexes were detected more frequently in patients with periodontitis than in healthy subjects [219]. Periodontitis has been reported to be associated with many systemic diseases[220], especially diabetes caused by metabolic syndrome. The prevalence of periodontitis in diabetic subjects was higher than that in non-diabetic subjects[221]. In addition, poorly controlled diabetic subjects have more severe periodontitis[222]. In periodontitis sites, a higher quantity of *P. gingivalis* in poor controlled diabetic subjects was observed than that of non-diabetic subjects[223]. Haffajee *et al*[224] reported that a reduction in the red complex was observed at sites where periodontal treatment with scaling and root planning alone or with systemically administered azithromycin, metronidazole, or a sub-antimicrobial dose of doxycycline was performed. In another study, Duarte *et al*[225] also found a reduction in red and orange complexes after periodontal treatment. Therefore, members of the red complex are still considered periodontal pathogens; however, some inconsistency exists in this concept. Previous studies demonstrated that *Streptococcus mutans* and *Streptococcus salivarius*, which were not defined as periodontal pathogens in the complex-based classification, causing attachment loss of periodontal tissues in animal studies[226,227]. Another example of contradictory findings was that periodontal bacterial species in a complex-based classification were detected, but periodontitis was not observed [218].

Recently, high-throughput DNA sequencing technology using next-generation sequencing (NGS) methods has been used to comprehensively analyze bacterial composition in different environments

[228]. In addition, bioinformatic techniques and pipelines have been improved to analyze data obtained from NGS[229]. In particular, NGS technology is suitable for bacterial analysis because the 16S rRNA genes from bacteria critically define all bacterial species and have an approximate length of 1 kbp of nucleotide sequences that are highly conserved among bacterial species[230]. Therefore, NGS technology combined with bioinformatic techniques is a useful tool for scientists as it allows for a comprehensive evaluation of microbial composition within the microbiome. Griffen *et al*[231] compared the bacterial composition of subgingival plaque between patients with chronic periodontitis and periodontally healthy controls using 16S pyrosequencing. They reported that the proportion of red complex microbes was high at the periodontitis sites, but the microbiome included bacterial species that were not previously regarded as being involved in the etiology of periodontitis. This result showed that conventional methods, such as culture methods, real-time polymerase chain reaction, or checkerboard DNA-DNA hybridization, were limited by the number of bacteria that could be detected. However, current methods using NGS are not limited to the number of bacterial species that can be detected. A few studies using NGS with the 16S rRNA gene region suggested that several taxa, including *Filifactor alocis* and TM7, showed greater abundance in periodontitis and seemed to act as the core bacteria in the subgingival microbiome of disease in addition to the bacteria thought to be conventional periodontal pathogens[231,232]. In other words, these results suggest that bacteria other than conventional periodontal pathogens, such as red complex members, also contribute to periodontitis, and dysbiosis within the microbiome potentially induces periodontal inflammation.

Hajishengallis *et al*[241] proposed the "keystone-pathogen hypothesis," wherein a specific bacterial species is considered a keystone periodontal pathogen, such as *P. gingivalis,* which changes the microbial balance and induces inflammation through dysbiosis, despite the low abundance of the pathogen[19,233]. Several problems with DNA-based analyses alone have indicated that the 16S rDNA profile may include dead bacteria[234]. On the other hand, only viable bacteria can be analyzed by conducting experiments using 16S rRNA[235]. However, analysis of only 16S rRNA is not a reliable index for bacterial activity and may be misleading in some cases because the relationship between rRNA and growth rate differs significantly in subspecies, even if these bacteria belong to the same bacteria at the species level[236]. Therefore, the 16S rRNA/16S rDNA ratio has been useful as a bacterial activity index for detecting highly active bacterial species, which, despite the low abundance of the pathogen, can induce dysbiosis in certain environments[237,238]. Kachi *et al*[237] clarified that the activity of *P. gingivalis* was lower than that of other species in periodontitis sites, although *P. gingivalis* was abundant in both DNA and RNA. In contrast, *T. forsythia*, *Fusobacterium nucleatum* subsp. *Vincentii, Streptococcus oralis, T. denticola, F. alocis, and Streptococcus salivarius* showed high activity in periodontitis sites. By analyzing both DNA and RNA using the 16S rRNA region, it may be easier to detect keystone pathogens because this analysis can lead to the understanding of not only the abundance of each bacterium but also their activities. However, these analyses alone cannot reveal the functional composition of the microbiome. Tools such as Piphillin or Piecrust can predict the functional composition of the microbiome using the information of the 16S rRNA region[239,240]. Ikeda *et al*[241] reported that pathways for phenylpropanoid and GPI-anchor biosynthesis and metabolism of alanine, arginine, aspartate, butanoate, cyanoamino acid, fatty acid, glutamate, methane, proline, and vitamin B6 were significantly over-represented in periodontitis subjects compared with healthy subjects using Piphillin. Thus, we might understand the essence of polymicrobial disease by determining not only the composition of the microbiome but also the functional gene composition.

In terms of bacterial composition analysis, the 16S rRNA region is highly conserved among prokaryotes, such as bacteria and archaea, and the length of the 16S rRNA region is suitable to provide sufficient information for phylogenetic analysis[230]. However, the length of the 16S rRNA region is almost 1600 base pairs. Among bacteria, *Mycoplasma*, which is the smallest parasitic bacterium, has 580000 base pairs[242]. This also indicates that the 16S rRNA region alone is insufficient for a complete understanding of the microbiome. In addition, 16S rDNA and 16S rRNA sequencing has caused PCR bias[243] and the different results depend on primer type[244]. To compensate for these shortcomings, metagenomic and metatranscriptomic analyses, which decode all DNA and RNA information, have attracted attention. Both metagenomic and metatranscriptomic analyses can analyze bacterial and functional composition, but metagenomic analyses focus more on species-level profiling, metatranscriptomic analyses concentrate more on gene expression levels[245]. A report by Dabdoub *et al*[246], using metagenomic analysis, reported the microbial and functional differences between healthy and periodontal sites. *Porphyromonas, Fusobacterium, Fretibacterium, Filifactor, Parvimonas, Selenomonas, Treponema,* and *Kingella* were more abundant in periodontitis sites than in healthy sites. In addition, most functional genes in healthy sites were dedicated to energy utilization through oxidative pathways, while fermentation and methanogenesis were the predominant energy transfer mechanisms in the disease site[246]. A study using metatranscriptomic analysis reported that *T. forsythia* and *P. gingivalis* upregulated different TonB-dependent receptors, peptidases, proteases, aerotolerance genes, iron transport genes, hemolysins, and clustered regularly interspaced short palindromic repeats-associated genes in periodontitis sites. Interestingly, bacteria that have not been usually implicated in periodontitis, such as *S. oralis*, *S. mutans*, *S. intermedius*, *Streptococcus mitis*, *Veillonella parvula*, and *Pseudomonas fluorescens*, were highly active in transcribing putative virulence factors in periodontitis sites[247]. Another report using metatranscriptomic analysis revealed that bacteria, such as *Bacteroides massiliensis*

and Leptotrichia hofstadii had high transcriptional activity in addition to that of the red complex[8]. Furthermore, combining metagenomic and metatranscriptomic analyses help understand the activity or enrichment of a given gene set in the microbiome[245]. The combination of metagenomic and metatranscriptomic analyses by Duran-Pinedo *et al*[248] showed that *P. gingivalis, T. forsythia, and T. denticola* exhibited a higher mean abundance and gene expression in periodontitis sites than in healthy sites. However, *Bacteroidetes* oral taxon 274, *Corynebacterium matruchotii,* and *L. hofstadii* in periodontitis sites showed a decrease in relative abundance but represented a high proportion of gene expression, when comparing healthy and periodontitis sites. In addition, the vast majority of virulence factors upregulated in periodontitis sites originate from bacteria that are not considered major periodontal pathogens, such as *Neisseria flavescens*, *C. matruchotii, Rothia dentocariosa*. Komatsu *et al*[249] combined metagenomic and metatranscriptomic analyses with a network analysis for periodontitis. Their results showed that *Fusobacterium nucleatum* subsp. *vincentii* had the highest activity in periodontitis sites, followed by *Peptostreptococcus stomatis* and *Leptotrichia* sp. according to the RNA/DNA ratio which are similar to the 16S rRNA/16S rDNA ratio. Additionally, *Fretibacterium fastidious,* and *F. alocis*, *Eubacterium nodatum* had high activity and were core bacteria in a co-occurrence network of periodontitis sites. In addition, the virulence factors, such as translation initiation factor IF-1 and pillin subunit in metatranscriptomic data were more enriched than in metagenomic data, indicating these virulence factors have high activity in periodontitis sites. These results suggest that there are bacteria that are more active than the red complex in periodontitis sites, those bacteria are involved in the pathogenesis of periodontal disease, and bacterial activity and interaction with each other are changed in response to environmental changes.

Periodontitis is not an infectious disease caused by a single bacterium, such as diphtheria, tetanus, typhoid fever, and leprosy, but a dysbiotic disease caused by alterations in the abundance of keystone and/or accessory pathogens within the polymicrobial community, leading to inflammatory responses (Figure 1). In addition, dysbiotic microbiota further develops and stimulate inflammatory responses[6]. However, it is difficult to comprehensively understand the etiology of periodontitis, although exhaustive DNA and RNA analyses have been performed. There are several possible explanations for this reason. First, many microorganisms, including bacteria, unculturable bacteria, fungi, and viruses, are present in the oral cavity[250]. Second, the same bacteria can act as homeostatic commensals in one environment and accessory pathogens in another. Furthermore, some bacteria can act as keystone or accessory pathogens depending on their condition[6]. Recently, trans-omics has been attracting attention and has focused on connecting multi-omics data, including not only the genome and transcriptome, but also the proteome and metabolome[251]. Trans-omics has been shown to be effective in elucidating various complex mechanisms, such as insulin sensitivity in liver cells and muscle adaptation[252,253]. These techniques might be applied to unravel the full picture of periodontal disease as a polymicrobial disease.

CHARACTERISTICS OF GUT MICROBIOME IN PATIENTS WITH PERIODONTAL DISEASE

Periodontal disease is characterized by a complex relationship between the host inflammation and polymicrobial dysbiosis of the oral-dental biofilm. This leads to chronic inflammation of the periodontal tissue, ultimately leading to bone destruction and tooth loss. However, over the last several years, this dysbiosis of the oral microbiome has been also associated with multiple systemic inflammatory conditions, such as cardiovascular disease, obesity, metabolic syndrome, and rheumatoid arthritis[71,254,255]. As mentioned previously, there are two main prevailing hypotheses regarding the potential for localized chronic periodontal infection to cause systemic inflammation. Namely: (1) Periodontal dysbiosis and inflammation leads to increase bacterial translocation into the systemic circulation causing inflammatory mediators and immune complexes to be circulated to other organ systems[256,257], and (2) Chronic periodontal dysbiosis leads to disturbances and changes to the gut microbiome *via* oral ingestion of periodontopathic organisms.

The concept that the oral microbiome drives the gut microbiome space is highlighted by the fact that some of the first bacteria to colonize the gut come from the mouth[258]. Infants born from vaginal deliveries are exposed to the microbiome of the mother's vaginal tract and gastrointestinal tract during labor, while breastfeeding introduces the skin microbiome to the infant[258]. As the gut microbiome matures over time, new bacterial strains are introduced into the gut *via* the environment and foods that we are exposed to in our childhood and young adulthood.

Yet, the established gut microbiome of adults is not stagnant, but rather can be change drastically by the environment, food, and/or medications. The connection of the oral microbiome and periodontal inflammation causing changes in the gut microbiome is highlighted in several animal and human studies. In a study of 44 patients, which included 7 healthy controls, authors found that patients with either gingivitis (n = 14) or chronic periodontitis (n = 23) had lower gut bacterial diversity as compared to healthy controls[19]. Specifically, they saw that patients with chronic periodontitis had higher levels of *Firmicutes, Proteobacteria, Verrucomicrobia* and *Euryarchaeota* and lower levels of Bacteroidetes as compared to controls[19]. In patients with gingivitis, *Prevotella, Comamonadaceae* and *Lactobacillales* were

elevated and *Bacteroidales* was decreased as compared to healthy control[19]. Utilizing a random forest classifier, *Mogibacteriaceae, Ruminococcaceae,* and *Prevotella* were able to discriminate individuals with periodontal diseases from healthy controls with an accuracy of 84%. Because of the cross-sectional design of this study, causality could not be inferred. However, several animal models have shown that the oral administration of *P. gingivalis* into mice leads to significant alteration of the gut microbiome, increase in serum endotoxemia, and a reduction of gut barrier function[20,32]. The oral administration of *P. gingivalis* in mice led to increases in *Bacteroidetes* and a reduction of *Firmicutes*[29,31]. These results in mice were recently mirrored in a translational study in humans. Bao *et al*[18] took salivary and fecal samples from 16 healthy participants and 21 patients with severe periodontitis and transplanted their salivary microbiome into wildtype C57BL6 mice. The authors showed that patients with severe periodontitis had a significantly different gut microbiome than those of healthy controls, with more saliva-sourced microbiome found in the gut of patients with severe periodontitis as compared to controls[18]. When transplanted into mice, the authors showed that by using utilizing carboxyfluorescein diacetate succinimidyl ester-stained bacteria the salivary microbiota persist in the gut for at least 24 h[18]. They also showed that the salivary microbiome of patients with severe periodontitis when transplanted into mice lead to an increase in gut levels of *Porphyromonadaceae* and *Fusobacterium*, while at the same time leading to a decreased expression of gut epithelial tight junction proteins and increased levels of pro-inflammatory cytokines. These findings alongside our prior works[11,22], demonstrate the important role of the oral-gut axis in other systemic diseases.

RELATIONSHIP BETWEEN GUT DYSBIOSIS AND NAFLD

Because of the interplay between the gut microbiome and inflammation, many studies have examined the role of the gut microbiome in liver disease. As part of this review, we will only focus on the relationship between the gut microbiome and NAFLD. The term NAFLD encompasses a spectrum of diseases that range from bland steatosis or NAFL to NASH and advanced hepatic fibrosis or cirrhosis. Several studies have linked gut dysbiosis to each phase of NAFLD.

NAFL

There have been several studies linking the gut microbiome to NAFL with variable results. In adult patients with NAFL, the most common genera that have been elevated were *Lactobacillus* and *Escherichia,* and *Coprococcus* and *Prevotella* were the most common to be reduced as compared to weight matched controls[259-262]. While these studies looked only at composition, several others examined NAFL utilizing a multiomics approach, which combined sequencing data with metabolomics. Raman *et al*[43] have shown that 18 stool metabolites, including increased levels of acetic acid, butanoic acid, and propanoic acid, are associated with NAFL in adult patients. Da Silva *et al*[261] also found a characteristic enrichment of isobutyric acid and propionic acid in the feces of NAFL patients. However, one of the most convincing data that has shown the causative relationship between the gut microbiome and NAFL comes from the translational study performed by Hoyles *et al*[262]. Utilizing hepatic transcriptome, gut metagenome, and serum and urine metabolome, they saw NAFL was associated with an increased serum level of several branched-chain and aromatic compounds, including phenylacetic acid[262]. In addition, administration of phenylacetic acid into mice transplanted with human fecal microbiota caused hepatic steatosis[262].

NASH

While studies examining the profile of NAFL were slightly mixed based on the population being investigated, studies examining the profile of NASH to obese controls showed more consistent findings[263]. In adults, NASH patients had lower levels of *Faecalibacterium, Ruminococcus and Bifidobacterium*[261,264] and a higher level of *Lactobacillus*[265]. Few studies have examined the metabolomics of NASH patients separate from NAFL, likely due to the reason that a diagnosis of NASH requires a liver biopsy to confirm. However, in one study of 16 adults diagnosed with NASH by biopsy, NASH patients had an increased ratio of primary to secondary bile acids which correlated with increase hepatic injury and inflammation[266].

NAFLD-related Advanced Fibrosis.

Advanced fibrosis is defined as a fibrosis stage of 3 or more and is associated with higher risk for morbidity and mortality. In general, microbiome studies of NAFLD-related advanced fibrosis usually see a decrease in microbial diversity marked by an increase in gram-negative bacteria[267-270]. Studies have found that NAFLD-related fibrosis was associated with an increase level of *Bacteroides* and *Escherichia*[267-269] with mixed results with other genera like *Prevotella*[268,270,271]. Metabolite studies found that 3-phenylproanoate[270] and 3-(4-hydroxyphenyl) lactate[272] were associated with NAFLD-related advanced fibrosis. 3-(4-hydroxyphenyl) lactate was also strongly correlated with several bacterial species that were also associated with hepatic fibrosis, such as *Escherichia coli*, *Bacteroides caccae* and *Clostridium sp*[272].

The mechanisms by which the gut microbiome can cause steatosis, inflammation, and hepatic fibrosis is highlighted in animal models. Patients with NAFLD and obesity are associated with a microbiome that leads to decreased gut epithelial barrier function[273]. This disruption in the gut epithelium leads to increased bacterial translocation, higher levels of endotoxemia, and subsequent increased activation of toll-like receptor signaling[274,275]. However, a clinical trial utilizing toll-like receptor inhibitor did not show any benefit in patients with NASH[276]. Other mechanisms, include alterations of short-chain fatty acids[277] and bile acid metabolism[278] and signaling[279].

PERIODONTAL APPROACHES IN THE PREVENTION AND TREATMENT OF NAFLD

Management of oral microbiota with conventional periodontal treatment

Periodontal treatment is based on the mechanical and chemical removal of dental plaque and pathogenic factors from the root surfaces by the patient or by a specialist to eliminate inflammation in the periodontal tissues and promote wound healing in the host. It is known that patients who achieve good oral hygiene and a healthy morphology of periodontal tissues through periodontal treatment show a decrease in the total number of bacteria and the proportion of periodontal pathogens in saliva and plaque, as well as a change in the composition of the oral microbiota to a healthy state. Therefore, given the relationship between periodontal disease and NAFLD supported by many epidemiological studies, periodontal therapeutic interventions may be a preventive measure for NAFLD (Figure 2).

In a cross-sectional study of 6352 adults participating in the Korean National Health and Nutrition Examination Survey, Kim *et al*[280] reported that there was an inverse association between frequency of tooth brushing and NAFLD prevalence (fatty liver index ≥ 60), with a modified odds ratio of 0.56 for NAFLD in the group that brushed three or more times a day compared with the group that brushed once a day or less. A few intervention studies have examined the effect of periodontal treatment on NAFLD. Yoneda *et al*[36] reported that in a single-arm intervention study, non-surgical periodontal therapy, including oral hygiene instruction and scaling/root planing (SRP) to 10 patients with NAFLD who had 4 or more sites with periodontal pockets ≥ 5 mm led to a significant improvement in AST and ALT at 3 mo after treatment. Interestingly, a recent multicenter randomized controlled trial by Kamata *et al*[38,281] demonstrated the efficacy of periodontal therapy for patients with NAFLD and periodontal disease. The control group (n = 20) received oral hygiene instruction only, while the experimental group (n = 20) received oral hygiene instruction and SRP. Serum levels of ALT and anti-*P. gingivalis* immunoglobulin antibody titers at 12 and 60 wk after treatment were measured as primary endpoints. Results showed significant reductions in ALT levels, endotoxin levels, and liver fat content in the SRP group compared to the toothpaste group. In addition, the decrease in *P. gingivalis* immunoglobulin G (IgG) antibody titer was significantly higher in the SRP group than in the toothpaste group. These decreased ALT levels and *P. gingivalis* IgG-antibody titers in the SRP group were sustained until 60 wk from baseline. The SRP group showed significantly greater reductions in blood lipid parameters, including total cholesterol, low density lipoprotein cholesterol, and triglycerides at 12 wk post-treatment, while no significant changes in glycometabolism parameters such as glucose and insulin.

Several studies presented in this section support the potential effect of periodontal treatment in improving outcomes in NAFLD. They also strengthen the causal relationship between periodontal disease and NAFLD. However, only one high-quality randomized controlled trial has been reported to date to assess treatment efficacy, and the relationship between the two is not clear. In the future, more evidence will need to be accumulated, and these controlled trials will need to be systematically meta-analyzed.

Oral and gut microbiome-targeted therapy using probiotics, prebiotics and bacteriocins

If opportunistic pathogens and periodontopathic bacteria that predominate in oral microbiome changes cause subsequent gut microbiome dysbiosis, an approach of microbiome-targeted therapy with antibiotics, probiotics and prebiotics may help prevent the onset and progression of NAFLD for patients with periodontal disease[11,22] (Figure 2). Probiotics are defined as live cultured microorganisms that provide health benefits in humans and animals when consumed, generally by improving or restoring the gut microbiota. While, prebiotics are defined as compounds in food that induce the growth or activity of beneficial microorganisms such as bacteria and fungi by altering the composition of organisms in the gut microbiome. When probiotics and prebiotics are used in combination, this is referred to as symbiotics. On the other hand, antibiotics exert beneficial effects on metabolic disorders by non-specifically suppressing the microbiome but may be accompanied by harmful side effects and potential emergence of antibiotic-resistant bacterial strains. Thus, currently, supplementation with probiotics and prebiotics in the treatment of NAFLD and periodontal disease has been favorably accepted due to a potential improved safety for humans and the environment[282-284].

As for treatment of NAFLD, multi-strain probiotic VSL#3 (contains eight species of *Streptococcus thermophilus*,*Bifidobacteria* [*B. breve*, *B. infantis*, *B. longum*], *Lactobacillus acidophilus*, *L. plantarum*,*L. paracasei* ,*L. bulgaricus*) was originally developed to treat inflammatory bowel disease, but VSL#3 administration has also been reported to improve fatty liver and fibrosis for NAFLD[285-287]. A randomized controlled

trial by Eslamparast *et al*[288] in patients with NAFLD showed that compared to the placebo group, a symbiotic consisting of a probiotic cocktail similar to VSL#3 and fructo-oligosaccharides given twice daily for 28 wk significantly improved liver function, inflammation, and fibrosis grade. Furthermore, a systematic meta-analysis of 21 randomized clinical trial concluded that the consumption of probiotics or symbiotics in NAFLD patients is an effective treatment modality to improve liver function, liver hardness, and blood markers of liver damage[282].

The effect of probiotics on periodontal treatment have also been reported to have several benefits in animal and clinical studies[289]. In studies using probiotics as monotherapy or as adjunctive therapy, *Lactobacillus salivarius* WB21, *L. plantarum, L. reuteri*, *L. rhamnosus* SP1, *Bacillus lactis*, and various *Streptococci* showed some efficacy in suppressing periodontopathic bacteria or other anaerobic bacteria. In particular, some *Lactobacillus* spp. have been noted to play a protective role against both NAFLD and periodontal disease[290-292]. Despite limited, beneficial effects were also observed in improving clinical parameters of periodontal disease, such as plaque score, gingival inflammation, and host immune response factors.

In recent years, our research team has reported multiple times that the use of nisin, an antimicrobial peptide produced by *Lactococcus lactis*, is effective in treating periodontal disease[289,293-296]. Nisin is a type of bacteriocin that is widely used in the food industry as well as in the medical field because of its potent and broad-spectrum antimicrobial activity even in small amounts, despite its low cytotoxicity and low incidence of bacterial resistance[297-299]. Nisin is classified as a Class I bacteriocin, and is a lantibiotic (lanthionine-containing antibiotics) based on its chemical structure, because it has abnormal amino acids that are caused by translational modifications[300-302]. With regard to the mechanism of action as an antimicrobial substance, nisin attaches to lipid II present in the bacterial cell membrane, and eight molecules of nisin form a tunnel-like pore with four molecules of lipid II, thereby promoting the outflow of intracellular ions, adenosine triphosphate and other substances from the bacteria[303-306]. The production of bacteriocins, such as nisin, is strictly regulated by bacterial signaling *via* quorum sensing, which works together with the self-tolerance capacity of bacteriocin-producing bacteria to selectively control the bacterial flora[307].

Interestingly, we found that in artificial oral biofilms derived from saliva, administration of the nisin-producing probiotic *L. lactis* or nisin significantly inhibited the biofilm formation and bacterial viability, reducing periodontal pathogens while maintaining endogenous oral bacteria such as *Neisseria* spp[294]. Similarly, wild type *L. lactis* or nisin dose-dependently reduced the viability of artificial biofilms formed on titanium alloy discs mimicking peri-implantitis and shifted the composition, relative abundance, and diversity level of the biofilms toward a healthy state with improved proportions of *Proteobacteria* and *Firmicutes* phylum[295]. Furthermore, in a polymicrobial mouse model of periodontal disease infected with multiple periodontopathic bacteria, we have shown that oral administration of nisin-producing *L. lactis* or nisin suppressed periodontal inflammation and alveolar bone loss[293]. These microbial treatments improved oral dysbiosis and decreased serum antibody responses to periodontal pathogens. On the other hand, non-nisin-producing *L. lactis* also reduced the oral and systemic impacts of the periodontal polymicrobial infection, but its effects were not as pronounced as those of nisin-producing *L. lactis*. Surprisingly, nisin and the *L. lactis* probiotic also induced a reparative phenotype *in vivo* in this polymicrobial disease model of periodontal disease. These results suggest that the combined interaction of living bacteria and their bacteriocins is important for probiotic use to be effective.

However, the significance of microbiome-targeted therapy in the treatment of periodontal disease-related NAFLD has been studied to a limited degree. In this regard, we are currently exploring the prophylactic effects of nisin on periodontitis-induced gut dysbiosis and liver disease using the polymicrobial infection mouse model. Detailed analysis of NAFLD-related genes and glycolipid metabolism in the liver is still ongoing in our lab, but we found that nisin shifts the oral and gut microbial composition to a healthy state and mitigates inflammatory responses. Nisin may also prevent hepatic lipid deposition by regulating mitochondrial functions and oxidative stress production in liver tissue.

Few studies have evaluated the effects of *L. lactis* probiotics or nisin in NAFLD, but some evidence based on *in vivo* experiments has been reported[308-310]. Lee *et al*[309] (2020) showed that oral administration of *L. lactis* NZ3900 strain pre-stimulated with nisin significantly protected fatty liver formation and progression of early atherosclerosis in a rabbit model fed a high cholesterol diet. In a study by Naudin *et al*[310] (2020) using mice fed a high calorie Western diet, compared to control mice taking the beneficial bacterium *Lactobacillus rhamnosus* GGL, oral administration of *L. lactis* subsp cremoris improved glucose tolerance and reduced serum cholesterol levels, weight gain, and obesity, resulting in less hepatic lipid deposition and inflammation. Jena *et al*[308] also reported that *Lactococcus* plays a protective role against liver inflammation in mice whose intestinal environment has been compromised by a Western diet. Although human studies on this topic are very limited, Ansari *et al*[311] showed that a fermented herbal formula containing *L. lactis* effectively improved serum liver function markers and liver fat deposition.

In summary, the use of probiotics, prebiotics, and bacteriocins may be a potential therapeutic strategy for patients with periodontal disease and NAFLD. These microbiome-targeted therapies have been shown to be safe for human health and easy to use on a daily basis in an environmentally friendly manner, so they may be applied alone or in combination with periodontal therapy. The fact that adjuvant therapy with probiotics in periodontal treatment has already been applied clinically will also

support the practical application of this new strategy. However, the development of microbiome-targeted therapies for NAFLD is in its infancy and further elucidation is warranted regarding the effectiveness of microbiome-targeted therapies on host immunomodulatory mechanisms, efficient intestinal delivery methods, effective combinations of various probiotics and bacteriocins, and methods for long-term maintenance of microbial composition and functional changes.

CONCLUSION

A growing body of evidence from multiple areas proposes that periodontal disease, accompanied by oral inflammation and pathological changes in the oral microbiome, induces gut dysbiosis and is involved in the pathogenesis of NAFLD[11,22] (Figure 1). The emerging concept of periodontitis-associated oral dysbiosis strongly implicates the role of the oral-gut-liver axis in the pathogenesis of NAFLD, based on the close relationship between the gut and liver connected by the enterohepatic circulation. This is due to the advancement of innovative technologies that enable comprehensive exploration of the microbiota through the widespread use of next-generation sequencing, which has fundamentally changed the definition of periodontopathic bacteria that has persisted in periodontology, and now highlights a turning point in rethinking the relationship between periodontal disease and systemic disease.

The liver is not merely a passive organ; NAFLD itself enhances insulin resistance and increases the risk of diabetes, atherosclerosis, and myocardial infarction with exacerbation of metabolic syndrome [312-314]. This means that even if NAFLD does not lead to end-stage liver disease, such as cirrhosis or cancer in individuals with metabolic syndrome, it can promote extrahepatic complications that can lead to significant mortality-related disease. Thus, both periodontal disease and NAFLD are interrelated with various elements of metabolic syndrome, leading to a vicious cycle of systemic inflammation and metabolic disturbance through amplification of these disease triangles[315]. Furthermore, in recent years, the liver from patients with NAFLD, as in the elderly and post-cardiac arrest donors, is a marginal organ that increases the risk of poor prognosis after liver resection or liver transplantation, the main treatment modalities for liver disease[316,317]. The detailed pathomechanisms of hepatic ischemia-reperfusion injury and post-transplant rejection in these operations are not known, but periodontal disease may be a potential risk factor for these hepatotoxic events as well[316,318-320].

Thus, oral and gut microbiome-targeted therapy based on management of periodontal infections would hold great promise for preventing the onset and progression of NAFLD and subsequent complications (Figure 2). Studies suggesting the efficacy of conventional periodontal treatment and microbiome-targeted therapy in patients with periodontal disease and NAFLD are increasing each year, but these are still in their infancy. Most reports of the relationship between periodontal disease and gut microbiome are based on data from animal studies, and a few studies have been conducted in humans. However, given the high prevalence of periodontal disease in recent years and the global increase in NAFLD, this new approach appears to be of great clinical and public health importance. In the future, long-term, large sample size cohort studies and randomized controlled trials should be conducted on this topic, in addition to further accumulation of rigorously controlled basic research.

FOOTNOTES

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Country/Territory of origin: United States

ORCID number: Ryutaro Kuraji 0000-0002-7900-3604; Takahiko Shiba 0000-0002-8388-6868; Tien S Dong 0000-0003-0105-8063; Yvonne L. Kapila 0000-0003-1330-0654.

Corresponding Author's Membership in Professional Societies: The Japanese Society of Periodontology; The International Association for Dental Research.

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REVIEW

Contributions of the receptor for advanced glycation end products axis activation in gastric cancer

Armando Rojas, Cristian Lindner, Iván Schneider, Ileana González, Miguel Angel Morales

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Armando Rojas, Ileana González, Biomedical Research Laboratories, Medicine Faculty, Catholic University of Maule, Talca 34600000, Chile

Cristian Lindner, Iván Schneider, Medicine Faculty, Catholic University of Maule, Talca 34600000, Chile

Miguel Angel Morales, Molecular and Clinical Pharmacology Program, Institute of Biomedical Sciences, University of Chile, Santiago 8320000, Chile

Corresponding author: Armando Rojas, PhD, Full Professor, Senior Researcher, Biomedical Research Laboratories, Medicine Faculty, Catholic University of Maule, 3605 San Miguel Ave., Talca 34600000, Chile. arojasr@ucm.cl

Abstract

Compelling shreds of evidence derived from both clinical and experimental research have demonstrated the crucial contribution of receptor for advanced glycation end products (RAGE) axis activation in the development of neoplasms, including gastric cancer (GC). This new actor in tumor biology plays an important role in the onset of a crucial and long-lasting inflammatory milieu, not only by supporting phenotypic changes favoring growth and dissemination of tumor cells, but also by functioning as a pattern-recognition receptor in the inflammatory response to *Helicobacter pylori* infection. In the present review, we aim to highlight how the overexpression and activation of the RAGE axis contributes to the proliferation and survival of GC cells as and their acquisition of more invasive phenotypes that promote dissemination and metastasis. Finally, the contribution of some single nucleotide polymorphisms in the RAGE gene as susceptibility or poor prognosis factors is also discussed.

Key Words: Gastric cancer; Advanced glycation end-products; Receptor of advanced glycation end-products; Alarmins; *Helicobacter pylori*; Chronic inflammation

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Core Tip: During the last two decades, new evidence supported by basic and clinical research has supported the role of the receptor for advanced glycation end products (RAGE) axis as a crucial actor in gastric carcinogenesis. We herein discuss how RAGE overexpression and RAGE activation-mediated signaling mechanisms are the main contributions of the RAGE axis to tumor development, migration, and metastasis in gastric cancer.

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INTRODUCTION

Gastric cancer (GC) is the fifth most common cancer and the fourth leading cause of cancer death worldwide[1]. It remains one of the most lethal neoplasms, with a 5-year survival rate of around 20%[2].

Although GC incidence and mortality rates have declined worldwide during the last decades, it still remains a very important clinical and public health issue. Important research efforts have shed new light not only on the main associated risk factors for development of GC, but also to the identification of crucial mechanistic contributors to gastric carcinogenesis[3-7]. Among these newly defined contributors to tumor growth and development of many cancer types, the receptor for advanced glycation end products (RAGE) multiligand axis has gained the attention of many research groups due to the diversity of mechanistic contributions to cancer growth and development supported by the complex signaling of the axis[5,8-11].

This review primarily highlights how the RAGE axis is involved in gastric carcinogenesis, focusing on its early contribution to the onset of a chronic inflammatory condition at the gastric epithelium and its role in tumor growth and development.

EPIDEMIOLOGY OF GC

A stated above, GC is the fourth most common cancer and the fourth leading cause of cancer death worldwide[1], despite the improvement of public health policies throughout the world to decrease the incidence and mortality rates from GC[12,13]. International reports have shown that the annual number of new GC cases is estimated to increase by 62% by 2024, especially in geographic areas of high-burden disease such as Asia, Eastern Europe, and South America[14]. The main risk factor for GC is infection by the bacterium *Helicobacter pylori* (*H. pylori*), due to this microorganism's ability to induce chronic inflammation in the gastric mucosa, atrophy of gastric glands, gastric intestinal metaplasia (IM), and carcinogenesis[14,15]. *H. pylori* infection is associated with 60%-80% of GC cases, as evidenced by extensive cohort studies of high-risk populations[16,17].

It is estimated that *H. pylori* infection is currently present in more than half of the world's population (4.4 billion people)[18]. Strikingly, only a small fraction of these *H. pylori*-infected individuals develop GC, with incidence ranging from 2% to 5%[19], strongly suggesting that GC development cannot only be explained by *H. pylori* infection. Accordingly, we must consider the role of different contributors to the sustained chronic inflammation observed in *H. pylori*-induced gastric carcinogenesis[5,20].

INFLAMMATION AND GC

In the 19th century, Rudolf Virchow outlined the putative connection between inflammation and cancer [21]. At present, there is compelling evidence supporting the role of chronic inflammation in organ-specific carcinogenesis, as is observed with *H. pylori*-induced gastric inflammation and the development of GC, gastric mucosa lymphoma[22], prostatitis and prostate cancer[23], chronic cholecystitis and gallbladder carcinoma[24], acid reflux–induced esophageal adenocarcinoma[25], and hepatocellular neoplasms associated with chronic viral hepatitis[26].

These data have increased our understanding of how chronic inflammation can promote not only the initiation of malignant transformation, but also the sculpting of the tumor microenvironment, and its transformation into a tumor-supportive niche. Through these mechanisms, inflammation can promote proliferation and survival of malignant cells, cancer cell acquisition of invasive phenotypes, dysregulation of the antitumoral immune response, and cancer cell resistance to chemotherapeutic agents[27,28]. The International Agency for Research on Cancer has recognized the bacterium *H. pylori* as a class 1

carcinogen[29]. In addition, recent evidence supports *H. pylori's* causal role in the majority of cases of cardia- and non-cardia GC[17].

The persistent inflammation caused by chronic *H. pylori* infection in the gastric mucosal epithelium is a critical initiating factor in the multistep processes of gastric carcinogenesis, also known as Correa's cascade: (1) Chronic inflammation; (2) atrophic gastritis; (3) metaplasia; (4); dysplasia; and (5) adenocarcinoma[16].

H. PYLORI-MEDIATED INFLAMMATION

H. pylori infection incites an inflammatory reaction through myriad mechanisms in gastric epithelial cells and circulating immune cells recruited to the site of infection. Inflammation can be triggered by classical pathogenicity factors such as CagA, cagPAI, urease, and outer membrane proteins, which all play roles in nuclear factor-kappa B (NF-κB) activation[30]. Outer membrane adhesion proteins such as OipA and BabA are involved in the induction of cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-8. CagA can also induce a marked IL-8 production, which is crucial to the recruitment of inflammatory cells and reactive oxygen species (ROS) production. Additionally, the contribution of *H. pylori* to an inflammatory milieu is supported by its induction of cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase transcription, which lead to enhanced production of inflammatory mediators prostaglandin E2 and nitric oxide, respectively[31-33].

However, the earliest activation of proinflammatory pathways is associated with the sensing capacity of the innate immune response *via* pattern recognition receptors (PRRs), which can recognize evolutionarily-conserved structures found on pathogens, termed pathogen-associated molecular patterns (PAMPs)[34]. Of note, PRRs can also recognize many structures known as alarmins, or damage-associated molecular patterns (DAMPs), which are associated with cellular necrosis in the setting of noxious stimuli such as inflammation[35]. PRRs play a pivotal role in the inflammatory response to *H. pylori* infection by recognizing both PAMPs and DAMPs. A compelling body of evidence supports the relevance of several PRRs in the immune response to *H. pylori*, including Toll-like receptors, NOD-like receptors, RIG-I-like receptors, C-type lectin receptors, and more recently the RAGE[36,37].

THE RAGE AXIS

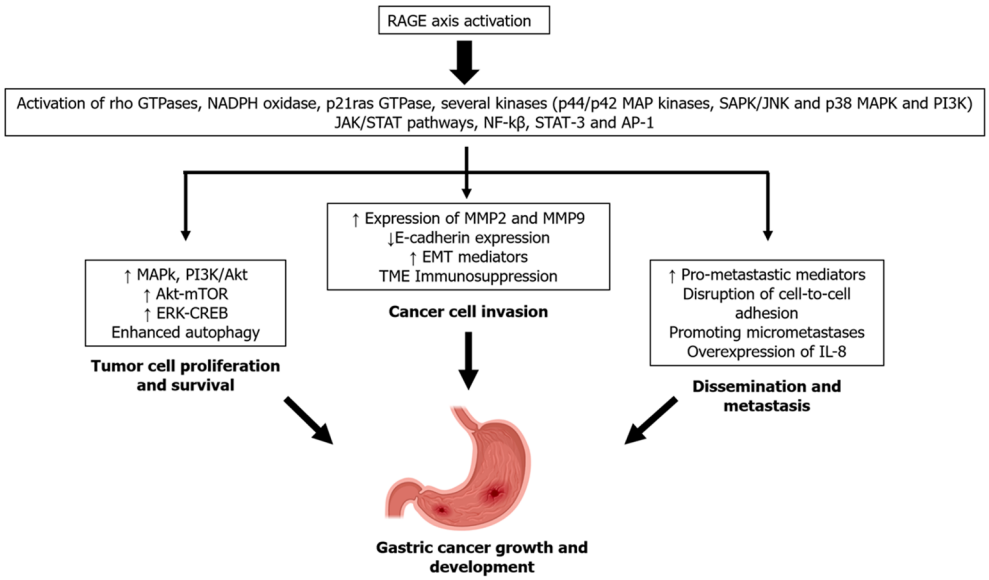
RAGE was initially recognized due to its ability to bind advanced glycation end products (AGEs). AGEs are a wide and diverse group of molecules derived *via* structural modifications of proteins, lipids, and nucleic acids, which all become non-enzymatically glycated by reducing sugars[38].

RAGE is a member of the immunoglobulin (Ig) superfamily. The human RAGE gene is located on chromosome 6p21.3, in the class II/III junction of the major histocompatibility complex locus and lies adjacent to the homeobox gene *HOX12*. The mature RAGE protein is 404 amino acids long with three major regions: an extracellular segment, a transmembrane segment, and an intracellular segment. The extracellular segment of RAGE is responsible for its ligand binding capacity and has three Ig domains, termed V-type, C1-type, and C2-type[39-42].

The integrated structural unit of the V and C1 domains is primarily responsible for interactions with a diverse group of negatively-charged RAGE ligands, including S100/calgranulins, AGEs, high mobility group box 1 (HMGB1) proteins, and Aβ-proteins[43]. The intracellular region of RAGE can also bind key molecules to activate downstream signaling, as reported in the cases of diaphanous-related formin 1 and toll-interleukin 1 receptor domain-containing adaptor protein (TIRAP)[44-46]. Soluble forms of RAGE, also known as soluble RAGE (sRAGE), have been identified and found in many fluid compartments, including serum[47,48]. These soluble variants arise from two main mechanisms: (1) Alternative splicing; and (2) enzymatic cleavage *via* the actions of matrix metalloproteinases (MMPs), disintegrin, and metalloprotease domain-containing protein 10[49]. RAGE activation by ligand binding triggers complex signaling cascades, leading to the activation of transcription factors such as NF-κB, signal transducer and activator of transcription 3 (STAT3), hypoxia-inducible factor-1α, activator protein-1, and cyclic-AMP response element binding (CREB). RAGE activation also stimulates p21ras, extracellular signal-regulated kinase (ERK) 1/2, p38 MAPK, stress-activated protein kinase/c-Jun n-terminal kinase, Rho GTPase, the PI3K and Janus kinase/STAT pathways. Accordingly, RAGE activation leads to a robust proinflammatory gene expression profile[50,51] (Figure 1).

Additionally, RAGE activation causes a positive feed-forward loop, where inflammatory stimuli activate NF-κB, which induces RAGE expression followed by sustained NF-κB activation[52].

The complexity of RAGE signaling is further expanded by its interaction at the plasma membrane with other receptors, such as the chemotactic G-protein-coupled receptors formyl peptide receptors 1 (FPR1) and FPR2, as well as the leukotriene B4 receptor 1[53]. Additionally, adaptor proteins for Toll like receptor 2 (TLR-2) and TLR-4 such as TIRAP and MyD88 also bind to the cytoplasmatic domain of RAGE, when phosphorylated at Ser 391 by PKC-zeta, following other ligand-mediated activation of RAGE[54].



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Figure 1 Receptor for advanced glycation end products engagement by several ligands such as advanced glycation end products, high mobility group box 1, S-100 calgranulin family proteins, and pathogen-associated molecular patterns from *Helicobacter pylori*. Receptor for advanced glycation end products (RAGE) activation by these various ligands triggers complex signaling cascades and consequently, the activation of a gene transcriptional profile that supports growth and development of gastric cancer. Soluble forms of RAGE (sRAGE) function as decoy receptors and do not activate RAGE signaling. Some adaptor molecules, such as mDia1 and toll-interleukin 1 receptor domain-containing adaptor protein, are required for optimal signaling. AP-1: Activator protein-1; CREB: Cyclic-AMP response element binding; EMT: Epithelial-to-mesenchymal transition; ERK: Extracellular signal-regulated kinase; *H. pylori*: *Helicobacter pylori*; IL-8: Interleukin-8; JAK: Janus kinase; JNK: c-Jun n-terminal kinase; MMP: Metalloproteinases; mTOR: Mechanistic target of rapamycin; NF-κB: Nuclear factor-kappa B; SAPK: Stress-activated protein kinase; STAT: Signal transducer and activator of transcription.

The gene encoding RAGE is highly polymorphic, and several single-nucleotide polymorphisms (SNPs) within this gene have been described. Compelling data support the role of RAGE gene polymorphisms in the onset and severity of several diseases, including cancer[55].

At present, RAGE is widely recognized as a PRR[56-58]. Beyond the AGEs, RAGE also acts a signaling receptor for various DAMPs or alarmins, such as members of the S100/calgranulin family, HMGB1, amyloid-beta (β) peptide, β-sheet fibrils, lysophosphatidic acid, Mac-1, phosphatidylserine, lipopolysaccharide, the complement component C3a, CpG oligos, and nucleic acids[59,60]. The emerging role of RAGE in sensing not only DAMPs but also PAMPs has been documented[61,62]. RAGE has also been reported to mediate the adherence of some pathogens to target cells by recognizing outer membrane proteins, as reported in the case of mycobacteria[63]. Of note, RAGE not only contributes to the adherence of *H. pylori* to gastric epithelial cells, but also to the production of the inflammatory response. This increase in RAGE expression and the amplification of the inflammatory cascade therefore culminates in *H. pylori*-induced gastric inflammation[64]. This finding is particularly interesting because this pathogen also uses another PRR, TLR-4, as a receptor for binding to gastric epithelial cells[65,66].

THE RAGE AXIS: AN EMERGING ACTOR IN TUMOR BIOLOGY

In the year 2000, an article published by Schmidt *et al*[67] outlined various RAGE axis contributions to pathophysiology. This study demonstrated its active participation in tumor biology, as the blockage of RAGE was shown to decrease tumor growth and metastases *in vivo*. Since then, additional compelling pieces of evidence have also demonstrated RAGE's contribution to carcinogenesis and have highlighted its expression levels on both tumor and stromal cells, the high diversity of signaling cascades triggered upon RAGE activation, its wide repertoire, and the abundance of RAGE ligands in the tumor microenvironment[68-70]. RAGE axis activation contributes to critical processes during tumorigenesis, such as the promotion of genetic instability, the initiation of chronic inflammation, the induction of phenotypic changes in tumor cells favoring growth and dissemination, and the support of an immunosuppressive environment[71,72].

Based on the growing body of evidence supporting the crucial role of the RAGE axis in many human pathologies, including cancer, and the availability of the 1.5 Å resolution crystal structure of the receptor, the RAGE axis has become an attractive target for the development of pharmacological interventions. Notably, the design and even the virtual screening of RAGE antagonists and signaling

inhibitors (*e.g.*, small-molecule inhibitors, natural products) has demonstrated the feasibility for inhibition of RAGE signal transduction, regardless of the nature of the ligand[51,73].

RAGE AXIS CONTRIBUTION TO GC

Tumor cell proliferation and survival

HMGB1 is a classical RAGE ligand with recognized roles in tumor biology. Of note, HMGB1 is overexpressed in gastric tumors, and a recent systematic meta-analysis demonstrated that HMGB1 plays an important role in GC, and its expression significantly correlates with tumor pT stage[74].

HMGB1-mediated RAGE activation triggers either MAPK or PI3K/AKT signaling and thus enhances tumor cell proliferation in different cancer cell types[75,76]. Additionally, data derived from *in vitro* experiments support the role of HMGB1 in promoting GC cell proliferation and migration *via* activation of RAGE-mediated Akt-mechanistic target of rapamycin (mTOR) and ERK-CREB signaling pathways, as well by initiation of a positive feedback loop that increases RAGE expression *via* these pathways. Furthermore, HMGB1 increases the expression levels of cyclin D1, cyclin E1, and proliferating cell nuclear antigen (PCNA), and HMGB1 knockdown reduces the expression of these proteins in GC cells [77].

The role in cancer cell proliferation of the S100 Calcium Binding Protein A16 (S100A16) remains controversial in some cancer types[78,79]. However, this alarmin is reported to promote cancer cell proliferation, migration, and invasion. *In vivo* and *in vitro* analyses revealed that S100A16 promotes both tumor cell proliferation and migration, whereas S100A16 knockdown abolished both[80]. Autophagy is a lysosome-mediated, self-degradation process that has emerged as key in protecting normal cells *via* the elimination of damaged organelles and protein aggregates and supporting bioenergetic homeostasis [81]. Autophagy can suppress the initiation of tumor development, or, in contrast, activate tumor cell survival, growth, and malignancy by facilitating access to metabolic demands during tumor progression and supporting tumor cell survival under stress[82-84].

The role of autophagy in GC progression, metastasis, and overall prognosis has been extensively documented[85,86]. Of note, HMGB1 released by autophagic GC cells through a RAGE-dependent mechanism acts as a pro-survival signal for remaining tumor cells, supporting cancer cell survival and promoting resistance to chemotherapy[87] (Figure 2).

Cancer cell invasion

Decades of research have demonstrated that the RAGE axis and its associated proinflammatory downstream pathways act as key mediators of carcinogenesis[88], especially in gastrointestinal tumor development[89].

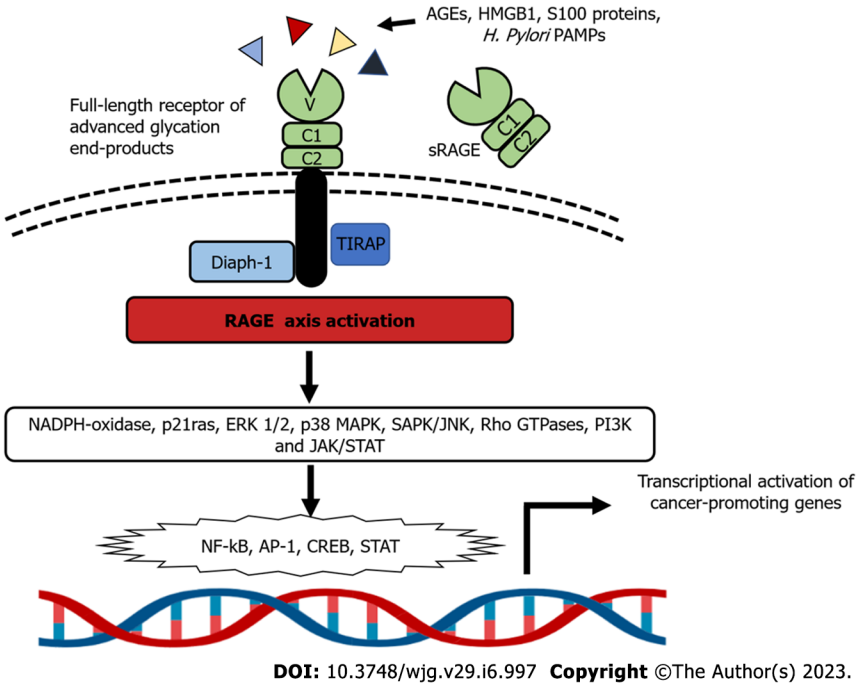
Among different cancer cell types, RAGE overexpression has been largely recognized as a pathological feature associated with increased tumor development, high invasiveness of cancer cells, and overall poor clinical prognosis[90-92].

The first experimental evidence supporting a correlation between RAGE overexpression and increased invasive and metastatic activity of GC cells was reported in 2002 by Kuniyasu *et al*[93]. This study revealed that RAGE axis signaling in gastric tumors may promote the acquisition of an invasive phenotype by increasing MMP-2 and MMP-9 expression, decreasing E-cadherin expression, and promoting tumor growth in a MAPkinase-dependent manner.

More recently, the analysis of RAGE expression in primary GC tissue reported by Wang *et al*[94] demonstrates that the upregulation of RAGE is associated with histological grade, nodal status, metastasis status, and American Joint Committee on Cancer (AJCC) stage in GC. RAGE upregulation is also associated with shorter overall survival rates. These data suggest that RAGE expression could be considered an independent predictor of GC aggressiveness and prognosis.

RAGE axis activation can have great influence on the invasive capacity of GC tumor cells through the modulation of cellular adhesion, motility, and production of MMPs. These functions are critical for epithelial-to-mesenchymal transition (EMT)[9,10], a complex process by which epithelial cells transdifferentiate to mesenchymal phenotypes. EMT is normally present during embryogenesis, tissue morphogenesis, wound healing, and regulation of stem cell behavior[95]. During this process, epithelial cells lose basal adhesion and demonstrate downregulation of epithelial cell markers (*e.g.*, E-cadherin) and upregulation of mesenchymal markers (*e.g.*, vimentin and fibronectin). These changes are also accompanied by increased expression of MMP-2 and MMP-9, which favors the degradation of ECM proteins[96]. EMT is a key mediator of the invasive and dissemination capacities of almost all types of cancer cells[97], including GC[98,99].

RAGE ligands have also been associated with cancer cell invasion and progression[100,101]. For example, the cancer-promoting role of HMGB1 has been extensively reported as a prognostic factor in many cancer types[74]. Importantly, differential expression analysis has shown that HMGB1 is increased in gastric adenocarcinoma cells[100] and has been directly associated with higher tumor stage, lymph node metastasis, depth of invasion, macrophage infiltration in the tumor microenvironment, and antineoplastic drug resistance[102-105]. Moreover, increased HMGB1 has also been correlated with poor



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Figure 2 Overview of RAGE-dependent intracellular signaling. RAGE binding triggers myriad mechanisms that support tumor proliferation and survival, invasion, and metastasis. AGE: Advanced glycation end product; AP-1: Activator protein-1; CREB: cyclic-AMP response element binding; ERK: Extracellular signal-regulated kinase; HMGB1: High mobility group box 1; JAK: Janus kinase; JNK: c-Jun n-terminal kinase; NF-κB: Nuclear factor-kappa B; PAMP: Pathogen-associated molecular patterns; RAGE: Receptor for advanced glycation end products; SAPK: Stress-activated protein kinase; STAT: Signal transducer and activator of transcription; TIRAP: Toll-interleukin 1 receptor domain-containing adaptor protein.

prognosis and overall survival in GC[106-109].

In vitro and *in vivo* assays have demonstrated that high concentrations of extracellular HMGB1 can directly impact the progression of GC cells through the induction of IL-8, which promotes tumor angiogenesis *via* increasing vascular endothelial growth factor (VEGF) and TNF-α expression[110]. Increased HMGBI also works to favor tumor invasiveness and promote EMT through RAGE-dependent activation of ERK 1/2 and NF-κB[111]. Recent experiments have also confirmed that HMGB1 activation of the RAGE axis can strongly regulate direct EMT mediators in GC cells *via* downregulation of E-cadherin, as well as by upregulation of transcription factors of Snail and Slug[111] and MMPs (*e.g.*, MMP-9 and MMP-2), whereas the knockdown of HMGB1 showed opposite effects. These results implicate HMGB1 is a key mediator of RAGE-dependent EMT, acting to favor the expression of more invasive GC cell phenotypes[77]. To this end, compelling data have revealed that RAGE knockdown in GC cells suppresses cell growth, invasion, and metastasis through the downregulation of RAGE-related pro-tumoral mediators of EMT (*e.g.*, Akt, PCNA, and MMP-2)[112]. Additionally, the knockdown of the *HMGB1* gene inhibits cell proliferation and the invasive potential of GC cells *via* suppression of RAGE-dependent NF-κB activation[113].

The S100 calcium-binding protein family member S100A8/A9 is a proinflammatory heterodimer produced by both myeloid-derived suppressor cells (MDSCs) and tumor cells[114]. S100A8 and S100A9 form a heterodimeric complex to interact with RAGE, promoting pro-tumoral signaling pathways within cancer cells[115]. Of note, serial analyses of gene expression reported significant overexpression of the genes encoding S100A8 and S100A9 in GC cells when compared to normal gastric epithelial cells [116]. In addition, *in vitro* evidence has revealed that these calcium-binding proteins are critical in promoting the invasive and migratory phenotype in GC cells through the engagement of RAGE and its pro-tumoral signaling pathways (*e.g.*, p38, MAPK, and NF-κB). These RAGE-activated pathways further mediate S100A8/A9-induced migration and invasion in GC[116,117]. For example, Yong and Moon[118] studied siRNA blockade of S100A8/A9 in a human GC cell line, and found a drastic decrease in the invasive ability of malignant cells. This finding suggests the critical role of S100A8 and S100A9 and their heterodimer in promoting progression and invasion in GC. Novel evidence has also shown a significant increase in MDSC-induced S100A8/A9 plasma levels in GC patients, which is thought to induce immunosuppressive effects through RAGE-dependent T-cell proliferation and interferon-γ production, which directly correlate with advanced cancer stage and reduced survival[119].

Interestingly, the emerging role of calprotectin as a modulator of apoptosis has been recently reported. Calprotectin has been shown to be involved in the regulation of apoptotic mediators such as Bax, Bcl-2 family proteins, and ERK signaling pathway proteins through RAGE engagement in GC cell lines[120].

Dissemination and metastasis

The dissemination of malignant cells from their primary site and the subsequent development of secondary tumors in distant organs, a process called metastasis, is a main consequence of cancer therapy failure and a major cause of cancer-related mortality[1,14]. The complex process of metastasis is supported by different mechanisms that require primary tumor cells to leave their origin, circulate within the microvasculature, escape immune surveillance, and colonize distant niches to promote the formation of secondary tumors[121].

A pivotal step in the development of secondary metastasis is the establishment of a complex interplay of tumor-derived factors, a favorable microenvironment in the distant site (*i.e.* a "pre-metastatic niche"). The pre-metastatic niche favors tumor cell colonization and promotes micro- and macrometastasis. Processes that contribute to the cultivation of the niche include inflammation, immunosuppression, neoangiogenesis, lymphangiogenesis, and organotropism[122].

Strikingly, the niche-promoting molecules described in various malignancies, including GC, are closely regulated by RAGE axis activation[71]. Examples of niche-promoting factors include S100A8/A9 ligand[39,123], VEGF-A, TNF-α, TGF-β, MMPs[43,46,124], extracellular vesicles, exosomes, and various microRNAs[5,7]; these mediators sculpt the cellular crosstalk that allows the initiation of metastatic secondary tumor formation[125,126]. In GC, it has been documented that patients with advanced stage and distant dissemination have a poor prognosis with a 5-year survival of approximately 20% depending on the population analyzed, tumor histological features, and the antineoplastic therapy used [1-3].

Experimental results suggest that RAGE overexpression is closely associated with the disruption of cell-to-cell adhesion and acceleration of GC cell motility, both of which support the metastatic activity of GC cells[93]. To this end, RAGE knockdown reduces GC cell proliferation and invasion and decreases the expression of pro-tumoral mediators (*e.g.,* Akt, PCNA, and MMP-2) consequently inducing aberrant cell apoptosis and cell cycle arrest[112]. For example, *in vivo* and *in vitro* evidence has shown that AGE binding to RAGE can accelerate gastric tumor progression and dissemination through the dose-dependent upregulation of the pro-tumoral mediators Specificity Protein 1 and MMP-2 *via* activation of ERK signaling[126,127].

In addition to its actions discussed above, HMGB1 also facilitates the progression and metastasis of various types of cancer[128], including GC[105,107]. In recent years, a growing body of evidence has begun to unravel the molecular mechanisms behind this contribution HMGB1 to CG metastasis[106]. For example, several studies have revealed increased expression of RAGE and HMGB1 in gastric tumor samples, which correlates with local and distant advanced disease and poor overall prognosis[102,107,109,110,113]. This association is accentuated under hyperglycemic conditions such as in diabetes mellitus, which enhance endogenous AGE formation and RAGE signaling[105]. Furthermore, other experimental research has shown that the overexpression of HMGB1 and the subsequent induction of IL-8 in the gastric tumor microenvironment (TME) may contribute to EMT and GC micrometastasis through the activation of downstream pathways of the RAGE axis, including ERK 1/2 and NF-κB[110]. In addition, overexpression of HMGB1 promotes the recruitment of neutrophils to the TME; this inflammation and associated production of ROS further promotes GC proliferation and metastasis[128,129].

Another recent investigation has demonstrated that the HMGB1/RAGE axis can regulate GC cell proliferation and migration *via* the enhanced expression of cyclins and MMPs, the induction of EMT, and activation of several cellular proliferation pathways including the Akt/mTOR and ERK/CREB signaling pathways[77].

Moreover, the *in vivo* or *in vitro* knockdown of HMGB1 leads to downregulation of NF-κB, PCNA, and MMP-9 expression in gastric tumor cells. This finding suggests that targeting the HMGB1 pathway may be a promising approach to inhibit the growth and metastasis of GC cells through the regulation of RAGE-dependent pathways[113]. Also, the overexpression of HMGB1 has been shown to enhance gastric tumor cell secretion of IL-8, supporting EMT during the early stages of tumor progression. Conversely, reduction in HMGB1/RAGE signaling by *in vivo* targeting of extracellular HMGB1 produces a significant reduction in tumor growth[111].

The overexpression of calgranulins in invasive GC cell lines has also been demonstrated[116]. These recognized RAGE ligands can promote not only more invasive phenotypes of the malignant cells, but can also enhance their migration capacity *via* RAGE-dependent mechanisms (*e.g.,* p38 MAPK-dependent NF-κB activation and MMP2 upregulation)[117].

RAGE polymorphisms

The expression level of RAGE has been associated with invasion, metastasis, and poor prognosis in GC [93,94]. RAGE levels also correlate with the severity of gastric mucosal lesions in patients infected with *H. pylori*, where the highest RAGE expression was observed in precancerous lesions (*e.g.*, atrophy or IM), dysplastic lesions, or *in situ* adenocarcinoma[130].

Compelling data have identified diverse polymorphisms within exons, introns, and regulatory regions of the gene encoding RAGE[131]. Interestingly, some of these polymorphisms may affect either RAGE gene transcriptional activity or the binding affinity of RAGE for its ligands[55,132]. Thus, RAGE polymorphisms are now potentially considered as susceptibility or poor prognostic factors in different cancer types. In this regard, several research groups have investigated the association between RAGE

Table 1 Main contributions of the receptor for advanced glycation end products axis to gastric carcinogenesis

Mechanism	Impact in gastric carcinogenesis	Year of publication	Ref.
RAGE overexpression	Promote tumor growth, migration and highly invasive phenotypes	2002	Kuniyasu *et al*[93]
	Association with high invasive histopathological grade, and poor overall survival	2015	Wang *et al*[94]
RAGE polymorphisms			
rs2070600	Association with increased risk of GC and highly invasive features	2008	Gu *et al*[138]
	Association with increased risk of GC	2017	Li *et al*[139]
rs184003	Association with decrease risk of GC	2017	Li *et al*[139]
RAGE activation			
HMGB1	Association with advance pT stage	2021	Zhou and Yang[74]
	Promotes GC cell proliferation and migration	2021	Tang *et al*[77]
	Support cancer cell survival and chemoresistance	2015	Zhang *et al*[86]
	Association to higher TNM stage, lymph node metastasis, and depth of invasion	2021	Zhou *et al*[105]
	Increased macrophage infiltration	2007	Akaike *et al*[103]
	Enhance tumor angiogenesis through induction of IL-8	2017	Chung *et al*[110]
	Promotes EMT activation and increased cell motility/invasiveness	2015	Chung *et al*[111]
	Promote GC progression *via* EMT	2020	Jin *et al*[104]
AGEs	Upregulation of pro-tumoral mediators	2017	Deng *et al*[127]
S100 proteins	Enhance tumor cell proliferation and migration	2021	You *et al*[80]
	Induced migration and invasion in GC cells	2013	Kwon *et al*[117]
	Promoting progression and invasion in GC cells	2007	Yong and Moon[118]
	Immunosuppressive RAGE-mediated effects	2013	Wang *et al*[119]
	Dysregulation of apoptotic factors	2020	Shabani *et al*[120]

AGE: Advanced glycation end products; EMT: Epithelial-to-mesenchymal transition; GC: Gastric cancer; HMGB1: High mobility group box 1; IL: Interleukin; RAGE: Receptor for advanced glycation end products.

gene polymorphisms and the risk of various cancers[133-137]. For example, studies have demonstrated the association of certain RAGE polymorphisms and GC. The pioneering work of Wang *et al*[136] demonstrated that the rs2070600 RAGE polymorphism [Gly82Ser] confers an increased risk of GC in the Chinese population[138]. Of note, this polymorphism is associated with enhanced RAGE signaling[132].

More recently, Liu *et al*[125] further confirmed that the rs2070600 variant AG genotype plays a predominant role in the development of GC. On the contrary, the rs184003 GT genotype represents a significantly reduced risk for GC. Additionally, the rs2070600 and rs184003 polymorphisms affect the serum levels of the splice variant of sRAGE, where the first is associated with a decreased level of while the latter with an increased serum level of sRAGE[139].

In addition to the protease-mediated cleavage soluble RAGE variants, sRAGE can arise from alternative splicing of the RAGE gene. These soluble variants lack the transmembrane and cytoplasmic domains, allowing them to function as decoy receptors, that do not lead to the activation of RAGE-associated signaling[46]. Reduced sRAGE has been associated with increased cancer risk and tumor progression in many other cancer types[140,141].

In summary, a diverse and compelling body of data shows the wide range of mechanistic contributions of the RAGE axis to gastric carcinogenesis (Table 1).

CONCLUSION

The RAGE axis is emerging as a relevant actor in gastric carcinogenesis. In this regard, a high expression level of RAGE and many RAGE ligands has been associated with tumor cell growth, invasion, metastasis, and poor prognosis. Furthermore, polymorphisms in the gene encoding RAGE have been linked to strengthening of RAGE ligand affinity, enhanced intracellular signaling, and modulation of

the serum levels of the soluble form of the receptor (sRAGE), which have been postulated as potential biomarkers of poor prognosis in GC patients.

However, additional research efforts are needed to fully understand the role of RAGE signaling in gastric carcinogenesis, particularly in those populations with a high prevalence of GC. Of note, increasing focus is being placed on diet and lifestyle modification as potential methods for preventing GC. This is particularly interesting, considering that in modern society, the consumption of AGEs is markedly increased (due in part to increased consumption of modern Westernized diets). Dietary intervention to restrict the intake of AGEs is recognized as a useful intervention, as demonstrated in several pathologies[10,11].

Today, clinical and experimental data demonstrate the therapeutic potential of blocking activation of the RAGE axis, as demonstrated by gene-silencing technologies, the use of aptamers, and the use of natural and synthetic molecules, all of which decrease RAGE ligand binding and/or RAGE-dependent intracellular signaling[51,70]. However, more clinical research is needed to establish the effectiveness of these promising options.

FOOTNOTES

Author contributions: All authors contributed to the original ideas and writing of this paper; Rojas A designed the report and wrote the paper; Lindner C created the artwork and performed data acquisition, drafting and revision of the manuscript; Schneider I, González I and Morales MA contributed to the data acquisition, drafting and revision of the manuscript.

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Country/Territory of origin: Chile

ORCID number: Armando Rojas 0000-0001-9911-7142; Cristian Lindner 0000-0002-2642-4288; Iván Schneider 0000-0001-5294-5995; Ileana González 0000-0002-2488-9380; Miguel Angel Morales 0000-0001-7698-9669.

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REVIEW

Implications of recent neoadjuvant clinical trials on the future practice of radiotherapy in locally advanced rectal cancer

Min Kyu Kang

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Min Kyu Kang, Department of Radiation Oncology, School of Medicine, Kyungpook National University, Daegu 41944, South Korea

Min Kyu Kang, Department of Radiation Oncology, Kyungpook National University Chilgok Hospital, Daegu 40414, South Korea

Corresponding author: Min Kyu Kang, MD, PhD, Associate Professor, Department of Radiation Oncology, Kyungpook National University Chilgok Hospital, 807 Hoguk-ro, Buk-gu, Daegu 40414, South Korea. mkkang@knu.ac.kr

Abstract

Over the last two decades, the standard treatment for locally advanced rectal cancer (LARC) has been neoadjuvant chemoradiotherapy plus total mesorectal excision followed by adjuvant chemotherapy. Total neoadjuvant treatment (TNT) and immunotherapy are two major issues in the treatment of LARC. In the two latest phase III randomized controlled trials (RAPIDO and PRODIGE23), the TNT approach achieved higher rates of pathologic complete response and distant metastasis-free survival than conventional chemoradiotherapy. Phase I/II clinical trials have reported promising response rates to neoadjuvant (chemo)-radiotherapy combined with immunotherapy. Accordingly, the treatment paradigm for LARC is shifting toward methods that increase the oncologic outcomes and organ preservation rate. However, despite the progress of these combined modality treatment strategies for LARC, the radiotherapy details in clinical trials have not changed significantly. To guide future radiotherapy for LARC with clinical and radiobiological evidence, this study reviewed recent neoadjuvant clinical trials evaluating TNT and immunotherapy from a radiation oncologist's perspective.

Key Words: Rectal cancer; Neoadjuvant therapy; Radiotherapy; Total neoadjuvant treatment; Immunotherapy

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Core Tip: In locally advanced rectal cancer (LARC), recent randomized controlled trials have demonstrated the benefits of total neoadjuvant treatment (TNT) in terms of oncologic outcomes and organ preservation. The results of clinical trials of immunotherapy suggest the possibility of pelvic radiotherapy in combination with immunotherapy for LARC. However, the radiotherapy details used in clinical trials have not changed significantly. This study reviewed recent neoadjuvant clinical trials evaluating TNT and immunotherapy from a radiation oncologist's perspective to guide future radiotherapy for LARC.

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INTRODUCTION

Neoadjuvant chemoradiotherapy (CRT) plus total mesorectal excision (TME) has been the standard treatment for locally advanced rectal cancer (LARC) for almost two decades. Although this approach has reduced the local recurrence (LR) rate to < 5%-10%, 20%-30% patients with LARC still experience distant metastasis (DM), a major cause of death[1-3]. Although the role of adjuvant chemotherapy (CT) on survival outcomes has been investigated by several working groups[4,5], the impact of adjuvant CT after neoadjuvant CRT plus TME on survival and ideal candidates for adjuvant CT are unclear[6]. Oxaliplatin-based neoadjuvant CRT, when compared to conventional CRT, showed higher toxicity rates without survival benefit in all phase III trials except one [7-10].

Total neoadjuvant treatment (TNT) and immunotherapy are the two trending issues in the preoperative treatment of LARC. Five phase III randomized controlled trials (RCTs) evaluating the TNT approach have been reported[11-15]. All trials showed significantly higher pathologic complete response (pCR) rates in the TNT group than in the traditional neoadjuvant CRT group. Moreover, two RCTs revealed that TNT significantly improved DM-free survival (DMFS), disease-free survival (DFS), or disease-related treatment failure[13,14]. Several prospective phase I/II studies have reported promising response rates to traditional neoadjuvant treatment combined with immune checkpoint inhibitors (ICIs)[16-19] or ICI alone[20] in LARC, known as an immune-cold tumor.

However, despite these advances in multidisciplinary treatment strategies for LARC, the radiotherapy (RT) details in clinical trials have not changed significantly. Therefore, to guide future RT for LARC with clinical and radiobiological evidence, this study reviewed recent neoadjuvant clinical trials evaluating TNT and ICIs from a radiation oncologist's perspective.

SUMMARY OF RECENT NEOADJUVANT CLINICAL TRIALS

TNT

The TNT approach brings the systemic adjuvant CT to the preoperative period, which can be administered before or after RT. The TNT approach has several clinical advantages in terms of tumor response, organ preservation, distant control, and long-term survival through early administration of intensive chemotherapeutic agents with a high compliance. The treatment protocols and important findings of RCTs evaluating TNT are summarized in Table 1 and Supplementary Table 1.

Three trials compared short-course RT (SCRT) followed by consolidation CT and standard CRT (POLISH II[21], RAPIDO[13], and STELLAR[15]). Between the SCRT + CT *vs* standard CRT groups, both RAPIDO and STELLA showed a higher pCR rate with TNT, which was not different in POLISH II. Notably, 3-year disease-related treatment failure and DMFS were significantly better in the TNT arm of RAPIDO, and 3-year overall survival (OS) was significantly better in the TNT arm of STELLAR. These results support the fact that SCRT followed by consolidation CT with a sufficient RT-to-surgery interval is a good option with a high pCR rate and potential to improve DMFS and OS, although there are differences in stage distributions, duration of consolidation CT, and RT-to-surgery intervals among these trials.

Three trials evaluated the role of induction or consolidation CT in TNT regimens (PRODIGE23[14], CAO/ARO/AIO-12[22,23], and OPRA[24]). PRODIGE23 showed that induction FOLFIRINOX (6 cycles) followed by standard CRT (*vs* standard CRT) was associated with better pCR, 3-year DFS, and 3-year DMFS rates. CAO/ARO/AIO-12 and OPRA reported no differences in DFS, LR, and DM between the induction and consolidation CT groups. However, the pCR rate in CAO/ARO/AIO-12 and 3-year TME-free survival in OPRA were significantly higher in the consolidation group than in the induction group.

Table 1 Recent randomized trials evaluating the total neoadjuvant treatment approach in locally advanced rectal cancer

Trials	Identifier	Patients	Primary endpoint	Treatment arms	Results (arm A *vs* B *vs* C)
POLISH II[11,21]	NCT00833131	cT4 or fixed cT3 (primary or locally recurrent)	R0 resection	A: CRT (5-FU+LV #2) → S (→ CT); B: SCRT → FOLFOX4 #3 → S (→ CT)	R0: 71% *vs* 77% (NS); pCR: 12% *vs* 16% (NS)
FOWARC[12]	NCT01211210	cT3-4 or cN+	3 yr DFS	A: CRT (de Gramont #5) → TME → de Gramont #7; B: CRT (mFOLFOX6 #5) → TME → mFOLFOX6 #7; C: mFOLFOX6 #4-6 → TME → mFOLFOX6 #6-8	pCR: 14.0% *vs* 27.5% *vs* 6.5%; 3 yr DFS: 72.9% *vs* 77.2% *vs* 73.5% (NS); 3 yr LF: 8.0% *vs* 7.0% *vs* 8.3% (NS); 3 yr OS: 91.3% *vs* 89.1% *vs* 90.7% (NS)
RAPIDO[13,109]	NCT01558921	cT4 or MRF+ or N2 or lateral LN+ or EMVI+	3 yr DRTF[1]	A: CRT (cape) → TME → CAPOX #9 or FOLFOX4 #12; B: SCRT → CAPOX #6 or FOLFOX4 #9 → TME	3 yr DRTF: 30.4% *vs* 23.7%[2]; 3 yr DM: 26.8% *vs* 20.0%[2]; pCR: 14% *vs* 28%[2]
PRODIGE23[14]	NCT01804790	cT3-4 Nany	3 yr DFS	A: CRT (cape) → TME → mFOLFOX6 #12; B: FOLFIRINOX #6 → CRT (cape) → TME → mFOLFOX6 #6	3 yr DFS: 69% *vs* 76%[2]; pCR: 12% *vs* 28%[2]
STELLAR[15]	NCT02533271	cT3-4 or cN+	3 yr DFS	A: CRT (cape) → TME → CAPOX #6; B: SCRT → CAPOX #4 → TME → CAPOX #6	3 yr DFS: 62.3% *vs* 64.5% (NS); 3 yr OS: 75.1% *vs* 86.5%[2]; pCR: 11.8% *vs* 16.6%[2]
CAO/ARO/AIO-12[22,23]	NCT02363374	cT3-4 or cN+	pCR	A: FOLFOX #3 → CRT (5-FU+oxaliplatin) → TME; B: CRT (5-FU+oxaliplatin) → FOLFOX #3→ TME	pCR: 17% *vs* 25%[2]
OPRA[24,110]	NCT02008656	cT3-4N0 or cTanyN1-2	3 yr DFS	A: mFOLFOX6 #8 or CAPOX #5→ CRT → WW or TME; B: CRT → mFOLFOX6 #8 or CAPOX #5 → WW or TME	3 yr DFS: 76% *vs* 76% (NS); 3 yr TME-free survival: 41% *vs* 53%[2]
Tang *et al*[68] ongoing	NCT04543695	Stage II-III with at least one high-risk factors: MRF+, T4, N2, lateral LN, EMVI+	Downstaging (yp0-II, cCR)	A: CRT → TME → CAPOX #6; B: CRT → CAPOX #6 → TME or WW; C: CAPOX #6 → CRT → TME or WW	(Preliminary) yp0-II: 77.1% *vs* 84.2% *vs* 57.1%; pCR + sustained cCR: 22.9% *vs* 42.1% *vs* 28.6%
CAO/ARO/AIO-18.1[76] ongoing	NCT04246684	MRI-defined intermediate and high-risk factors: cT3 low rectal (< 6 cm from AV), cT3c/d middle rectal (≥ 6-12 cm), cT4 tumors, Tany middle/low third of rectum with N+, CRM ≤ 1 mm, EMVI+	3 yr organ preservation	A: SCRT → CAPOX #6 or mFOLFOX6 #8 → WW or TME; B: CRT (5-FU+oxaliplatin) → CAPOX #4 or mFOLFOX6 #6 → WW or TME	

[1]Disease-related treatment failure was defined as the first occurrence of locoregional failure, distant metastasis, a new primary colorectal tumor, or treatmentrelated death.

[2]Denotes statistically significant differences.

5-FU: 5-fluorouracil; AV: Anal verge; cape: Capecitabine; cCR: Clinical complete response; CRM: Circumferential resection margin; CRT: Chemoradiotherapy; CT: Chemotherapy; DFS: Disease-free survival; DM: Distant metastasis; DRTF: Disease-related treatment failure; EMVI: Extramural vascular invasion; LF: Local failure; LN: Lymph node; MRF: Mesorectal fascia; MRI: Magnetic resonance image; NS: Not significant; OS: Overall survival; pCR: Pathologic complete response; S: Surgery; SCRT: Short-course radiotherapy; TME: Total mesorectal excision; WW: Watch-and-wait.

Although the TNT approach for LARC has already been adopted in several practice guidelines[25-28], a one-size-fits-all approach would not be appropriate in many real-world clinical scenarios. Based on the results of the aforementioned RCTs of TNT, Hui *et al*[29] and Roeder *et al*[30] introduced their institutional neoadjuvant approach for LARC according to the risk of local and/or distant failure and patients' desire for organ preservation.

Immunotherapy

Radiation has immunomodulatory effects on the host immune system in addition to the direct tumor cell killing effect[31,32]. In particular, dendritic cells activated by radiation can present tumor antigens in lymph nodes (LNs) and activate CD8+ T cells, which are important for killing primary or distant tumor cells[31,33,34]. However, upregulation of programmed death ligand 1 (PD-L1) expression in tumor cells by interferon-γ produced by CD8+ T cells can lead to radioresistance[35,36]. Thus, the potential of a combination of ICIs and RT has been investigated in various cancers, including LARC [37]. In addition, neoadjuvant treatment changes colorectal cancer to an immunogenic tumor *via* loss of

the mismatch repair (MMR) protein, decrease in mRNA expression levels of MMR-related genes, and increase in the tumor mutational burden (TMB)[38-40]. Several early-phase clinical trials have reported higher response rates to neoadjuvant treatment combined with ICIs for LARC, when compared to historical rates with conventional CRT. Their treatment protocols and important findings are summarized in Table 2 and Supplementary Table 1.

Four trials evaluated tumor response to CRT combined with concurrent and/or consolidation ICIs. Five cycles of consolidation nivolumab after CRT in the VOLTAGE-A phase I/II trial achieved pCR rates of 30% in patients with microsatellite-stable (MSS) and 60% in patients with high microsatellite instability (MSI-H)[16]. Two trials reported pCR rates after CRT with concurrent and consolidation ICIs; 23% in the AVANA phase II trial[17] using 6 cycles of avelumab and 24% in the R-IMMUNE phase Ib/II trial[18] using 4 cycles of atezolizumab. The NRG-GI002 phase II RCT compared two groups of induction mFOLFOX6 (6 cycles) followed by CRT with or without concurrent and consolidation pembrolizumab; the pCR rate was not different between the groups (31.9% *vs* 29.4%)[19]. Two phase II trials evaluating SCRT followed by consolidation CT and ICIs reported the highest pCR rates; 37.5% in the AVERECTAL trial[41] using 6 cycles of mFOLFOX6 plus avelumab and 48.1% in the trial by Lin *et al*[42] using 2 cycles of CAPOX plus camrelizumab. Notably, an ongoing TORCH trial reported a preliminary clinical complete response (cCR) or pCR rate of 81.3% in 16 MSS patients who received SCRT-based induction or consolidation CAPOX plus toripalimab[43]. In addition, these studies enrolled patients regardless of the MMR status, two of which reported the pCR rate according to the MMR status [16,42]. Although these promising results should be verified in large-scale RCTs, the results indicate the potential benefits of the combination of pelvic RT and ICIs in the aspect of organ preservation and survival outcomes, especially for patients with MSS LARC.

Despite the proportion of patients with deficient MMR (dMMR) or MSI-H LARC being as low as 5%-10%, neoadjuvant ICIs with or without CRT or SCRT for dMMR/MSI-H LARC are gaining attention owing to their high response rates[44]. Most recently, Cercek *et al*[20] preliminarily reported that all 12 consecutive patients with dMMR LARC achieved cCR after 9 cycles of dostarlimab and were under watch-and-wait (WW) without CRT, surgery, or any recurrence for 6-25 mo. Several ongoing trials are evaluating the response rate and toxicities of SCRT combined with ICIs in patients with dMMR/MSI-H LARC[45-47] (Table 2 and Supplementary Table 1).

RT in clinical trials

The RT dose, field, and techniques were similar among the TNT and immunotherapy trials described above (Supplementary Table 1). The clinical target volume (CTV) included the primary tumor, regional LNs, and pelvic regions at risk, as proposed by Roels *et al*[48] and the Radiation Therapy Oncology Group[49], as long as its definition could be confirmed in the protocol or published article. A total dose of 50-50.4 Gy at 1.8-2 Gy per fraction and 45 Gy at 1.8 Gy per fraction were administered to the gross tumor volume (GTV) and elective pelvic areas during CRT, respectively. All trials using SCRT administered 25 Gy in 5 fractions to the CTV. Some studies allowed an additional boost dose to the GTV (up to a total dose of 54-56 Gy). Intensity-modulated RT (IMRT) or 3-dimensional conformal RT (3D-RT) were used. Although the treatment outcomes of the aforementioned clinical trials using these traditional target volumes and RT doses are promising, better outcomes can be achieved if RT parameters can be optimized for TNT and immunotherapy. The factors to be considered when using RT as a part of TNT or in combination with ICIs will be discussed in later sections.

CLINICAL IMPLICATIONS

Paradigm shift of neoadjuvant treatment regiments

Neoadjuvant CRT (45-50.4 Gy in 25-28 fractions concurrently with 5-fluorouracil or capecitabine) has been recommended in LARC as it improves the resectability, sphincter preservation, and local control rates. Although SCRT (25 Gy in 5 fractions without CT) with delayed surgery is considered effective as CRT in terms of sphincter preservation, R0 resection, and local control, SCRT has not been widely used because of insufficient downstaging and high toxicity[50-52]. On the other hand, many efforts have been made to omit or selectively use pelvic RT in LARC to avoid RT-related toxicities[53,54]. LARC with a low risk of LR can be successfully treated without preoperative or postoperative pelvic RT[25]. However, these approaches require radical resection, which is associated with surgery-related complications. In addition, the WW strategy became doubtful when Habr-Gama *et al*[55] reported excellent long-term outcomes of nonoperative management for patients with LARC who achieved cCR after CRT (50.4 Gy with concurrent 5-fluorouracil and leucovorin) in 2004.

Currently, the neoadjuvant treatment paradigm is shifting in several ways owing to the excellent results of TNT trials[56]. TNT regimens combined with CRT or SCRT are recommended by the National Comprehensive Cancer Network guidelines[57] and are increasingly employed in clinical practice[56]. SCRT is regaining attention because of the following advantages when combined with TNT and immunotherapy: Short treatment time, high compliance, few side effects, and potential synergistic effects with immunotherapy. The WW strategy is being accepted in patients achieving cCR after

Table 2 Recent prospective clinical trials using immune checkpoint inhibitors as a component of neoadjuvant treatment for locally advanced rectal cancer

Trials	Identifier	Patients	Primary endpoint	Treatment	Results
VOLTAGE-A [16]	NCT02948348	cT3-4 Nany (regardless of MMR status)	pCR	CRT (cape) → Nivolumab #5 → TME (→ mFOLFOX6/CAPOX)	pCR: 30% for MSS, 60% for MSI-H
AVANA[17]	NCT03854799	cN+ or cT4 or high-risk cT3 (CRM ≤ 1 mm, ≤ 6 cm from AV, or T3c/d)	pCR	CRT (cape) + Avelumab #6 → TME	pCR: 23%
R-IMMUNE [18]	NCT03127007	Stage II or III	pCR, safety	CRT(5-FU) + Atezolizumab #4 → S	pCR: 24%
NRG-GI002 [19]	NCT02921256	Stage II or III with at least 1 of the following criteria: cT3-4 ≤ 5 cm from AV, any cT4 or tumor within 3 mm of MRF, cN2, not candidates for sphincter-sparing surgery at presentation	Neoadjuvant rectal score	A: mFOLFOX6 #6 → CRT (cape) → TME; B: mFOLFOX6 #6 → CRT (cape) + Pembrolizumab #6 → TME	NAR score: 14.08 *vs* 11.53 (NS); pCR: 29.4% *vs* 31.9% (NS)
AVERECTAL [41,111,112]	NCT03503630	cT2N+ or cT3-4aNany	pCR	SCRT → mFOLFOX6 + Avelumab #6 → TME	pCR: 37.5%
Lin *et al*[42]	NCT04231552	Stage II or III	pCR	SCRT → CAPOX + Camrelizumab #2 →TME	pCR: 48.1% (13/27), 46.2% (12/26) for pMMR, 100% (1/1) for dMMR
TORCH[43, 113] ongoing	NCT04518280	cT3-4 or cN+	cCR or pCR	A: SCRT → CAPOX + toripalimab #6 → TME or WW; B: CAPOX + toripalimab #2 → SCRT → CAPOX + toripalimab #4 → TME or WW	(Preliminary) cCR + pCR: 81.3% (13/16 MSS patients); group A (n = 7): cCR 1, pCR 4, near pCR 1; group B (n = 9): cCR 4, pCR 4
Cercek *et al*[20] ongoing	NCT04165772	cT3-4 or cN+ with dMMR or MSI-H	cCR, pCR, overall response	Dostarlimab #9 → if cCR → WW; if residual+ → CRT → WW (cCR) or TME (residual+)	cCR: 100%
PRIME-RT[45] ongoing	NCT04621370	cT3b+ or EMVI+ or CRM ≤ 2 mm or a low rectal tumor requiring abdomino-perineal excision	cCR or pCR	A: (SCRT → mFOLFOX6 #6) + durvalumab #4 → S or WW; B: (CRT → mFOLFOX6 #4) + durvalumab #4 → S or WW	
EA2201[46] ongoing	NCT04751370	cT3-4 or cN+ with dMMR or MSI-H	pCR	Ipilimumab/Nivolumab #2 → SCRT → Ipilimumab/Nivolumab #2 → TME	
Qiu[47] ongoing	NCT04636008	cT2 or higher with dMMR or MSI-H	Adverse reaction after neoadjuvant treatment and perioperative complications	SCRT + Sintilimab #3 → TME or WW	

5-FU: 5-fluorouracil; AV: Anal verge; cape: Capecitabine; cCR: Clinical complete response; CRM: Circumferential resection margin; CRT: Chemoradiotherapy; dMMR: Deficient mismatch repair; EMVI: Extramural vascular invasion; MMR: Mismatch repair; MRF: Mesorectal fascia; MSI-H: High microsatellite instability; MSS: Microsatellite stable; NAR: Neoadjuvant rectal; NS: Not significant; pCR: Pathologic complete response; pMMR: Proficient mismatch repair; S: Surgery; SCRT: Short-course radiotherapy; TME: Total mesorectal excision; WW: Watch-and-wait.

neoadjuvant treatment, owing to the accumulation of clinical evidence supporting the safety of WW [58]. Although there was no difference in DFS, LR, and OS between mFOLFOX6 with or without RT in FOWARC[12] (Table 1 and Supplementary Table 1), a higher pCR rate in patients receiving RT supports the use of pelvic RT with a higher chance of organ preservation. Even in early rectal cancer with cT2-3abN0, CRT or SCRT followed by local excision or WW has achieved excellent pCR or organ preservation rates[59,60]. These trends emphasize the importance of RT in future neoadjuvant treatment regimens for rectal cancers.

Organ preservation

There is a growing demand for organ preservation to avoid surgical complications or permanent colostomy, which translates into an improved quality of life after treatment for LARC. Various efforts have been made to increase the cCR rate[61]: Escalating the radiation dose, increasing the interval between RT and surgery, and adding consolidation CT before surgery[58,62,63]. When combined with conventional CRT, these approaches increase the rate of cCR, but not survival outcomes[64-66].

A meta-analysis revealed that the TNT approach showed a better pCR rate and survival outcomes than conventional CRT[67], suggesting that the TNT approach is a good option for organ preservation with survival benefits. Based on the results of CAO/ARO/AIO-12[22,23] and OPRA[24], consolidation CT is preferred when using CRT in the WW strategy. Preliminary results of a Chinese phase II RCT also showed the highest CR rate (pCR + sustained cCR) in the consolidation group (22.9% in the CRT group *vs* 28.6% in the induction group *vs* 42.1% in the consolidation group)[68].

Although data on the WW strategy after SCRT are limited, SCRT followed by consolidation CT appears to be an option for organ preservation based on the following evidence. SCRT followed by at least 4 cycles of consolidation CT was associated with significantly better pCR and DFS rates than conventional CRT in a meta-analysis[69]. Studies using SCRT followed by consolidation CT reported similar cCR, LR, and TME-free survival rates as those reported in studies using CRT[24,70,71]. Based on the results of a meta-analysis, at least 4 cycles of consolidation CT after SCRT should be considered[69]. However, the optimal duration of consolidation CT needs to be determined to avoid undertreatment or overtreatment.

Based on the high response rates reported in the AVERECTAL[41], Lin *et al*[42]'s, and TROCH[43] trials, despite the limited data on the role of immunotherapy combined with (C)RT, SCRT-based TNT plus ICIs seems plausible for the WW strategy in MSS LARC. In patients who cannot tolerate intensive CT, CRT with concurrent or consolidation ICIs may be an option for organ preservation, given that the pCR rate (23%-30%) of these approaches seems to be higher than that of traditional CRT[16-18]. The results of ongoing trials of SCRT combined with ICIs (Table 2 and Supplementary Table 1) are anticipated because hypofractionated RT has better immunostimulatory effects than prolonged RT; this will be discussed in a later section. Induction CT combined with ICIs is discouraged based on the results of NRG-GI002[19]. Extremely high response rates (up to 100%) to immunotherapy alone, shown in retrospective[72] and prospective[20] studies, support the use of immunotherapy alone for dMMR LARC. However, in dMMR LARC with high tumor burden, immunotherapy alone may be insufficient to eliminate all the tumor cells in the body when considering the association between tumor burden and the response to immunotherapy[73]; therefore, the combination of RT and immunotherapy may be needed for dMMR LARC. To provide individualized treatment, future trials should focus on identifying the optimal combination of RT, CT, and ICIs.

RT-RELATED ISSUES

Although the treatment outcomes of the aforementioned clinical trials using traditional target volumes and radiation doses are promising, the current practice of RT for LARC may not be optimal for the combination with TNT and ICIs. The following sections provide clinical and radiobiological evidence for the RT details that would be suitable for the combination therapies in LARC.

RT dose without immunotherapy

The current guidelines typically recommend the conventional long-course RT of 50-50.4 Gy in 25-28 fractions with concurrent CT as neoadjuvant treatment for LARC, with a total dose of ≥ 54 Gy in patients with unresectable tumors or patients who desire organ preservation[25,27,57]. Appelt *et al*[74] reported a positive dose-response relationship for pathologic tumor regression, and two meta-analyses revealed a high pCR rate of 24%-28% with a total dose of ≥ 54 Gy (mostly 1.8-2.2 Gy per fraction) with or without CT intensification[66,75]. However, because no dose-response relationship or survival benefit has been proven for a total dose > 54 Gy, the impact of such high doses is unclear[66,75]. In addition, surgical complications can occur more frequently with equivalent dose in 2 Gy fractions > 58.9 Gy[66]. Thus, total doses of 54-56 Gy with induction or consolidation CT have been used in studies with the intent of organ preservation in LARC[24,62,76].

Traditionally, a total dose of 25 Gy in 5 fractions has been used for SCRT as the sole neoadjuvant treatment for LARC, with a pCR rate of approximately 10%[77]. SCRT (25 Gy in 5 fractions) followed by consolidation CT achieved a higher pCR rate (17%-28%) than conventional CRT (12%-14%)[13,15]. A single institutional retrospective study reported that SCRT (25-30 Gy in 5 fractions) followed by FOLFOX or CAPOX achieved a cCR rate of 50%, persistent cCR rate of 79% in the WW cohort, and a 2-year TME-free survival rate of 40% (69% in patients with cCR)[71]. Although the same institute reported a high 1-year cCR rate of 70% in 19 patients who were treated with dose-escalated SCRT (30 Gy to the primary tumor and 35 Gy to the extramesorectal LNs in 5 fractions) followed by FOLFOX or CAPOX [78], further studies are warranted to evaluate the benefits and toxicities of radiation dose escalation in SCRT-based TNT due to a paucity of data in this area.

RT dose with immunotherapy

Recent prospective studies combining ICIs with RT (Table 2) used traditional dose-fractionation schedules in LARC: Conventional RT (45-50.4 Gy with 1.8-2 Gy per fraction) or hypofractionated RT (25 Gy with 5 Gy per fraction). Different immunological effects between conventionally fractionated and hypofractionated schedules have been reported, mainly in murine cancer models.

In a murine colon cancer model (CT26), conventional RT (5 × 2 Gy or 18 × 2 Gy) exerted immunosuppressive effects by upregulating PD-L1 expression in tumor cells and inducing immunosuppressive myeloid cells (myeloid-derived suppressor cells and tumor-associated macrophages 2)[36,79]. The negative impact on local tumor control and survival of mice was reversed by the addition of programmed death receptor-1 (PD-1)/PD-L1 inhibitors to fractionated RT. In addition, the combination of ICIs and CRT is supported by an increase in TMB, a known predictor of response to immunotherapy [80], in patients with LARC treated with neoadjuvant CRT[40,81]. Seo *et al*[40] reported that CRT decreased the mRNA expression levels of 23 MMR-related genes in LARC. Furthermore, loss of the MMR protein has been observed after neoadjuvant CRT for LARC[38-40,82]. These results explain the high pCR rates (23%-30%) of conventional CRT (45-50.4 Gy in 25-28 fractions) combined with ICIs in VOLTAGE-A[16], AVANA[17], and R-IMMUNE[18]. With insufficient data, it is unclear whether RT dose escalation in neoadjuvant CRT for LARC is beneficial.

Hypofractionated RT using a large fractional dose appears to be more efficacious when combined with ICIs, compared with conventional RT. Preclinical studies have reported that various schedules using 5-8 Gy per fraction elicited CD8+ T cell-mediated immune responses effectively in irradiated local tumors and non-irradiated distant tumors (the abscopal effect) when combined with various immunotherapeutic agents[34,79,83]. Dendritic cells had a higher homing ability to lymphoid tissues when they received an RT dose ≥ 5 Gy than when they received 10 × 2 Gy[84]. Radiation-induced lymphopenia is a known negative prognostic factor for tumor response or oncologic outcomes in various solid tumors, including rectal cancer[85-87]. Moreover, persistent lymphocyte depletion is associated with poor treatment outcomes after immunotherapy for various solid tumors[88]. Hypofractionated schedules was associated with less lymphocyte depletion or faster recovery after treatment in several tumor sites[89-92]; this supports the use of neoadjuvant SCRT in combination with immunotherapy over protracted conventional CRT. The higher tumor response rates of SCRT followed by consolidation CT plus ICI in prospective studies[41-43] (*vs* in trials of CRT plus ICIs[16-18]) may facilitate the use of SCRT in the neoadjuvant treatment regimen for LARC, although the exact causes of this difference cannot be explained, except by the difference in the use of consolidation CT. Nonetheless, since it is unknown whether a combination of the traditional regimen of 25 Gy in 5 fractions with immunotherapy is optimal, further studies are warranted to determine the optimal dose-fractionation regimen, especially in terms of dose escalation.

RT field

Traditional RT fields include GTVs and regional lymphatics with subclinical nodal metastases. When RT is combined with immunotherapy, elective nodal irradiation (ENI) is not recommended for the following reasons. First, irradiation to tumor-draining LNs can reduce RT-mediated immune responses. Tumor-draining LNs where cytotoxic CD8+ T cells are activated by dendritic cells, are important for RT-induced immunologic cell death[33,93]. In a murine melanoma model (B16F10), irradiation to tumors (1 × 10 Gy) and tumor-draining LNs (3 × 3 Gy) reduced the response of both irradiated and non-irradiated tumors compared to tumor-only irradiation[93]. In another murine colorectal carcinoma model (MC38), a single 12-Gy ENI reduced intratumoral infiltration of functional antigen-specific CD8+ T cells[94]. Second, severe lymphopenia, which has a negative effect on immunotherapy, can occur because of the large RT field that includes the elective nodal area[95,96].

Although a CTV including the primary tumor and mesorectum below the S2/S3 interspace was safe in patients with T2 low-lying/T3, N0-N1 rectal cancers without lateral LN metastasis in a prospective phase II trial[97], there is no clinical evidence against ENI in the neoadjuvant setting of LARC. Future studies should weigh the immunologic benefit of no ENI and risk of recurrence following undertreated metastatic LNs. Nevertheless, since the tumor response to whole pelvic RT combined with ICIs is promising in early-phase clinical trials (Table 2 and Supplementary Table 1), long-term patterns of failure and survival data are anticipated.

RT planning

For a long time, radiation oncologists have tried to lower radiation doses to normal pelvic organs to reduce acute and chronic gastrointestinal and genitourinary toxicities after pelvic RT. The importance of lymphocyte-sparing RT has recently emerged in the era of immunotherapy[98]. There are several reasons supporting the need of lymphocyte sparing during the neoadjuvant pelvic RT of LARC. Approximately one-quarter of the proliferating bone marrow of the body is in the pelvis[99]. Permanent aplasia in irradiated bone may occur with irradiations > 30 Gy[100]. Fatty changes in the pelvic bone marrow are a common observation after CRT[101]. Pelvic RT for pelvic malignancies can cause prolonged lymphocyte depletion[102,103].

In addition to the dose-fractionation schedule and target volume size, the pelvic bone marrow and lymphocyte count can be influenced by the dose rate and delivery techniques such as 3D-RT and IMRT. Although IMRT can reduce acute gastrointestinal and genitourinary toxicities in LARC[104,105], it is unclear whether IMRT is superior to 3D-RT in terms of sparing of active bone marrow (or lymphocytes) [86,95]. The estimated mean dose to the entire circulating blood pool after 60 Gy in 30 fractions for glioblastoma was similar for 3D-RT and IMRT with a sliding window technique (2.4 Gy and 2.7 Gy) [95]. Patients with LARC treated with preoperative pelvic RT with helical tomotherapy showed

significantly higher rates of post-treatment neutrophil-lymphocyte ratios ≥ 4 than those treated with 3D-RT[86]. The impact of delivery techniques such as volumetric-modulated RT (VMAT) is unclear, which can deliver radiation doses faster with less low-dose spillage than other IMRT delivery modes such as step-and-shoot and sliding window techniques[106]. Kuncman *et al*[107] reported an association between the dose of active bone marrow on magnetic resonance imaging and absolute lymphocyte count nadir and platelet nadir. Huang *et al*[108] reported a lower incidence of acute hematologic toxicity with pelvic bone marrow-sparing IMRT (IMRT with sliding window or VMAT) using dosimetric constraints for the pelvic bone marrow of V10 ≤ 85%, V20 ≤ 65%, and V30 ≤ 45% (VX = volume receiving X Gy) than with non-sparing IMRT in patients with LARC treated with CRT. Therefore, it is necessary to determine the optimal dose constraints for the pelvic bone (or bone marrow) and proper dose delivery techniques to enhance the effects of immunotherapy.

CONCLUSION

Recent phase II/III RCTs have confirmed the benefits of the TNT approach combined with conventional CRT or SCRT in terms of oncologic outcomes and organ preservation in LARC. The results of prospective phase I/II studies suggest the possibility of conventional CRT or SCRT in combination with immunotherapy for LARC. Preclinical and clinical evidence suggests the RT details which may be suitable for the neoadjuvant pelvic RT as a part of TNT or in combination with immunotherapy. However, further studies are warranted to identify optimal RT strategies with the potential of maximizing the effects of neoadjuvant treatment with TNT and immunotherapy.

FOOTNOTES

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Country/Territory of origin: South Korea

ORCID number: Min Kyu Kang 0000-0002-7962-7054.

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REVIEW

Therapeutic interventions target the NLRP3 inflammasome in ulcerative colitis: Comprehensive study

Fares E.M Ali, Islam M. Ibrahim, Osama M Ghogar, Esraa K. Abd-alhameed, Hanan S. Althagafy, Emad H.M. Hassanein

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Fares E.M Ali, Emad H.M. Hassanein, Pharmacology and Toxicology, Faculty of Pharmacy, Al-Azhar University, Assiut Branch, Assiut 71524, Egypt

Islam M. Ibrahim, Osama M Ghogar, Faculty of Pharmacy, Al-Azhar University, Assiut Branch, Assiut 71524, Egypt

Esraa K. Abd-alhameed, Pharmacology and Toxicology, Faculty of Pharmacy, Beni-Suef University, Beni-Suef 12345, Egypt

Hanan S. Althagafy, Department of Biochemistry, Faculty of Science, University of Jeddah, Jeddah 12345, Saudi Arabia

Corresponding author: Fares E.M Ali, MSc, PhD, Lecturer, Research Scientist, Pharmacology and Toxicology, Faculty of Pharmacy, Al-Azhar University, Assiut Branch, Al-Azhar Street, Assiut 71524, Egypt. faresali@azhar.edu.eg

Abstract

One of the significant health issues in the world is the prevalence of ulcerative colitis (UC). UC is a chronic disorder that mainly affects the colon, beginning with the rectum, and can progress from asymptomatic mild inflammation to extensive inflammation of the entire colon. Understanding the underlying molecular mechanisms of UC pathogenesis emphasizes the need for innovative therapeutic approaches based on identifying molecular targets. Interestingly, in response to cellular injury, the NLR family pyrin domain containing 3 (NLRP3) inflammasome is a crucial part of the inflammation and immunological reaction by promoting caspase-1 activation and the release of interleukin-1β. This review discusses the mechanisms of NLRP3 inflammasome activation by various signals and its regulation and impact on UC.

Key Words: Ulcerative colitis; NLR family pyrin domain containing 3 inflammasome; Therapeutic strategies; Phytochemicals; Probiotics

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Core Tip: Ulcerative colitis (UC) is a common chronic type of inflammatory bowel disease that affects a significant number of populations. Needing to counteract the UC prevalence, attract scientists to study its pathological mechanism deeply. NLR family pyrin domain containing 3 (NLRP3) inflammasome has been observed to have a crucial role in the pathological features of UC. Targeting NLRP3 inflammasome signals with phytochemicals, plants, probiotics, and chemical agents could be promising candidates for fixing the UC problem.

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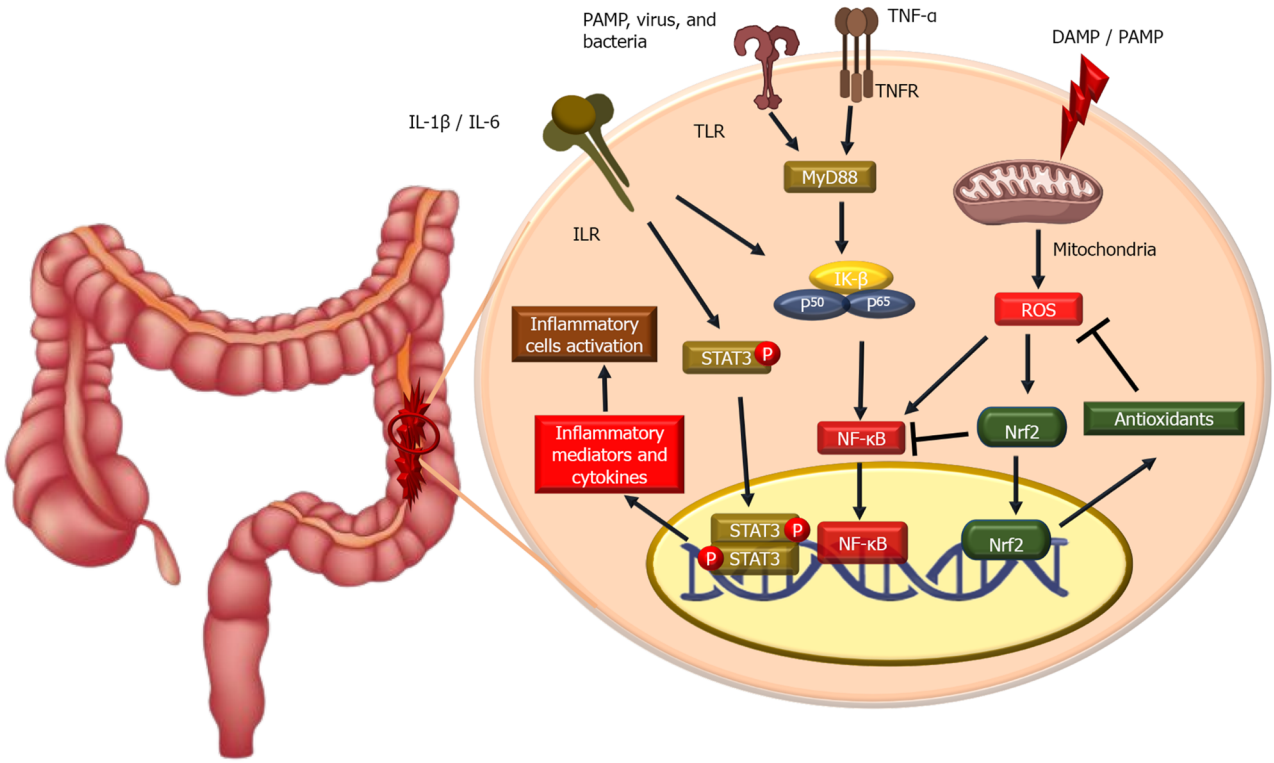
INTRODUCTION

Inflammatory bowel diseases (IBD), most referred to as Crohn's disease and ulcerative colitis (UC), are chronic, idiopathic disorders characterized by intestinal inflammation that have increased incidence and prevalence over time and in various countries worldwide[1-3]. The global prevalence is expected to impact up to 30 million by 2025[4]. Several factors are known to play a considerable role in disease pathogenesis. IBD has been linked to genetic etiology and other variables such as pathogens, appendectomy, stress, and air pollution[5,6]. UC is a chronic disorder that mainly affects the colon, beginning with the rectum and perhaps progressing to inflammation of the entire large intestine[7]. UC can begin with severe illnesses such as melena, diarrhea, and mucus production[8,9], and often between the ages of 50 years and 80 years[3,10,11].

A family history of IBD elevates the potential risk of UC, and first-degree relatives are four times more likely to acquire the disease, suggesting that genetic factors are likely related to UC[12,13]. Also, environmental factors play a crucial role in its development[14]. About 110 of the 163 susceptibility loci (67%) encode innate and adaptive immunity pathways, cytokine signaling, and immunological sensing. Several other autoimmune diseases, including psoriasis and ankylosing spondylitis, share many of these genes[15,16]. Additionally, many UC-specific genes have a role in controlling epithelial barrier function [17,18].

The gastrointestinal mucosa is susceptible to many antigens from food, the environment, and the microbiome[19]. The mucin layer that covers the epithelium, the mucosa's outer layer, serves as the gut immune system's first line of defense since it physically separates antigens from gut immune cells and has antimicrobial capabilities. Patients with active UC have been demonstrated to have a thinner mucin-containing mucosal layer in the colon, primarily due to a reduction in mucin 2 production[20]. In addition, exposure to potentially harmful stimuli such as nonsteroidal anti-inflammatory drugs and food components such as emulsifiers may prompt colitis[21,22]. Moreover, dysbiosis reduces short-chain fatty acid synthesis, which is required for epithelial energy supply and mucus formation[23]. On the same approach, UC has been attributed to a decrease in short-chain fatty acid-producing *Ruminococcaceae* and *Lachnospiraceae* and an increase in pro-inflammatory microorganisms such as Enterobacteriaceae[24,25].

When the epithelium is damaged, the mucosa becomes more permeable to luminal pathogens, increasing the absorption of these antigens and possibly activating gastrointestinal tract immunity[26,27]. Since it is the link between the host immune response repertoire and the intestinal microbiota, the intestinal epithelium plays a crucial role in the innate immune system[28]. Antigens activate the innate immune response *via* antigen-presenting cells and T cells, which evoke an inflammatory response that activates the adaptive immune system[27,29]. Mechanistically, mature dendritic cells express a lot of Toll-like receptors (TLR), which activate multiple transcription factors like nuclear factor-κB (NF-κB), which in turn causes an inflammatory response in UC[30,31]. These inflammatory cascades result in the production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukins (IL)[26,27,29]. These pro-inflammatory cytokines have critical roles in signaling through intracellular proteins, including Janus kinase (Jak), which enhances lymphocyte activation and proliferation[32,33]. Interestingly, innate immune signaling *via* cytokine receptors, TNF receptors, and TLR-adaptor myeloid differentiation as a primary response promotes NLRP3 transcription and oligomerization through NF-kB activation[34,35]. Figure 1 outlines this inflammatory response inside the colon in IBDs, including UC.



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Figure 1 Inflammatory response inside the colon in inflammatory bowel diseases including ulcerative colitis. Several inflammatory pathways are involved in ulcerative colitis (UC), which result in increased inflammatory mediators and cytokines and subsequent activation of inflammatory cells. First, activation of Toll-like receptors (TLR) by pathogen associated molecular pattern (PAMP), bacteria and viruses as well as tumor necrosis factor-alpha receptor (TNFR) stimulation by TNF-α, induce nuclear factor-κB (NF-κB) translocation through upregulation of MyD88 and IK-β, P50, and P65 complex. Second, along with NF-κB upregulation, interleukin receptor stimulation by interleukin (IL)-1β and/or IL-6 activates signal transducer and activator of transcription 3 signalling activation. Third, damage associated molecular pattern and/or PAMP are stimulates mitochondria to generates reactive oxygen species (ROS) which enhances inflammatory response through NF-κB activation whereas activating nuclear factor erythroid 2-related factor 2 signalling by ROS suppresses the inflammatory response by blocking NF-κB signalling and inducing antioxidants that downregulates ROS. IBDs: Inflammatory bowel diseases; UC: Ulcerative colitis; IL-(1β): Interleukin-1 beta; DAMP: Damage associated molecular pattern; PAMP: Pathogen associated molecular pattern; TNF-α: Tumour necrosis factor-alpha; ILR: Interleukin receptor; TLR: Toll-like receptor; TNFR: Tumour necrosis factor receptor; MyD88: Myeloid differentiation primary response 88; IK-β: Inhibitor *kappa*-beta; P50: Nuclear factor-kappa B P50 subunit; P65: Nuclear factor-kappa B P65 subunit; ROS: Reactive oxygen species; STAT3: Signal transducer and activator of transcription 3; NF-κB: Nuclear factor-kappa B; Nrf2: Nuclear factor erythroid 2-related factor.

NLRP3 INFLAMMASOME OVERVIEW

The innate immune system is the primary mechanism by which most organisms respond quickly to diseases or injury. Pattern-recognition receptors (PRRs) are activated by the host and identify chemicals released by infections or damaged cells. Pathogen-associated molecular patterns and damage-associated molecular patterns are the names of these molecular signals[36]. Numerous intracellular DNA sensors, TLRs, nucleotide-binding and oligomerization domain (NOD)-like receptors, C-type lectin receptors, RLRs, and NOD-inducible gene-I-like receptors are among the numerous members of the PRR family [37].

A multi-protein complex NLRP3 inflammasome is a type of PRR essential for the host's innate defenses against bacterial, fungal, and viral infections[38-41]. However, when it is dysregulated, it has been connected to the pathogenesis of many inflammatory-associated diseases, including cancer, neuroinflammation, retinopathy, stroke, diabetes, atherosclerosis, and autoinflammatory diseases[42-46]. The three protein components that make up NLRP3 are an amino-terminal pyrin domain (PYD), a NOD, and a leucine-rich repeat domain at the protein's C-terminus[47]. Apoptosis-associated speck-like protein (ASC) is composed of two proteins, pyrin and a caspase-recruitment domain (CARD), but their interaction to initiate inflammasome assembly facilitates it to recruit pro-caspase-1 to the inflammasome complex[48,49].

The activation of the NLRP3 inflammasome is essentially caspase-1 autocatalysis. Once turned on, NLRP3 functions as a sensor molecule that self-oligomerizes and recruits ASC through homotypic PYD-PYD interaction, causing ASC to assemble into sizable speck-like formations. Caspase-1 is then autocatalytically activated as aggregated ASC recruits pro-caspase-1 through CARD-CARD interaction. Proteolytic activation of the pro-inflammatory cytokines IL-1β and IL-18 and the soluble cytosolic protein gasdermin D (GSDMD) is the function of activated caspase-1 heterotetramers. Following

proteolysis, the oligomerized gasdermin N can bind membrane lipids and create membrane pores to mediate the release of IL-1β and IL-18 outside the normal secretory pathway. In parallel, cells go through pyroptosis, a pro-inflammatory cell death[50-53]. IL-1β stimulates endothelial cell response by allowing immune cell infiltration into infected or injured tissues *via* the activation of genes that regulate temperature, pain threshold, vasodilation, and hypotension[54]. IL-18 is a co-stimulatory cytokine necessary for mediating adaptive immunity through the influence of interferon-gamma production[54].

MECHANISMS OF NLRP3 INFLAMMASOME ACTIVATION

It is believed that several cellular signals, including ion fluxes (K^+ efflux, Cl^- efflux, Ca^{2+} influx, and Na^+ influx), mitochondrial dysfunction, and reactive oxygen species (ROS) generation, are responsible for NLRP3 inflammasome activation[55-60]. In addition, heme[61,62], particulate matter[56,63,64], pathogen-associated RNA[65-68], and bacterial and fungal toxins[69,70] are also considered NLRP3 activators.

NLRP3 activators require a priming signal (signal 1) to be activated[71]. TLRs, NOD-like receptors (*e.g.*, NOD1 and NOD2), or cytokine receptors such as TNF-α and IL-1β, bind to stimuli in the priming signal, which is crucial for macrophage stimulation. Pro-IL-1β, which is not constitutively produced in inactive macrophages, is upregulated by NF-κB. In addition, NF-κB also upregulates the expression of NLRP3, which is assumed to exist in quantities insufficient for starting inflammasome activation during rest[71,72]. In contrast, priming signals are not crucial for ASC, pro-caspase-1, and pro-IL-18 production levels[71]. Inflammasome activation and NLRP3 self-association are regulated by the priming signal's induction of Jun N-terminal 1-mediated phosphorylation of NLRP3[73].

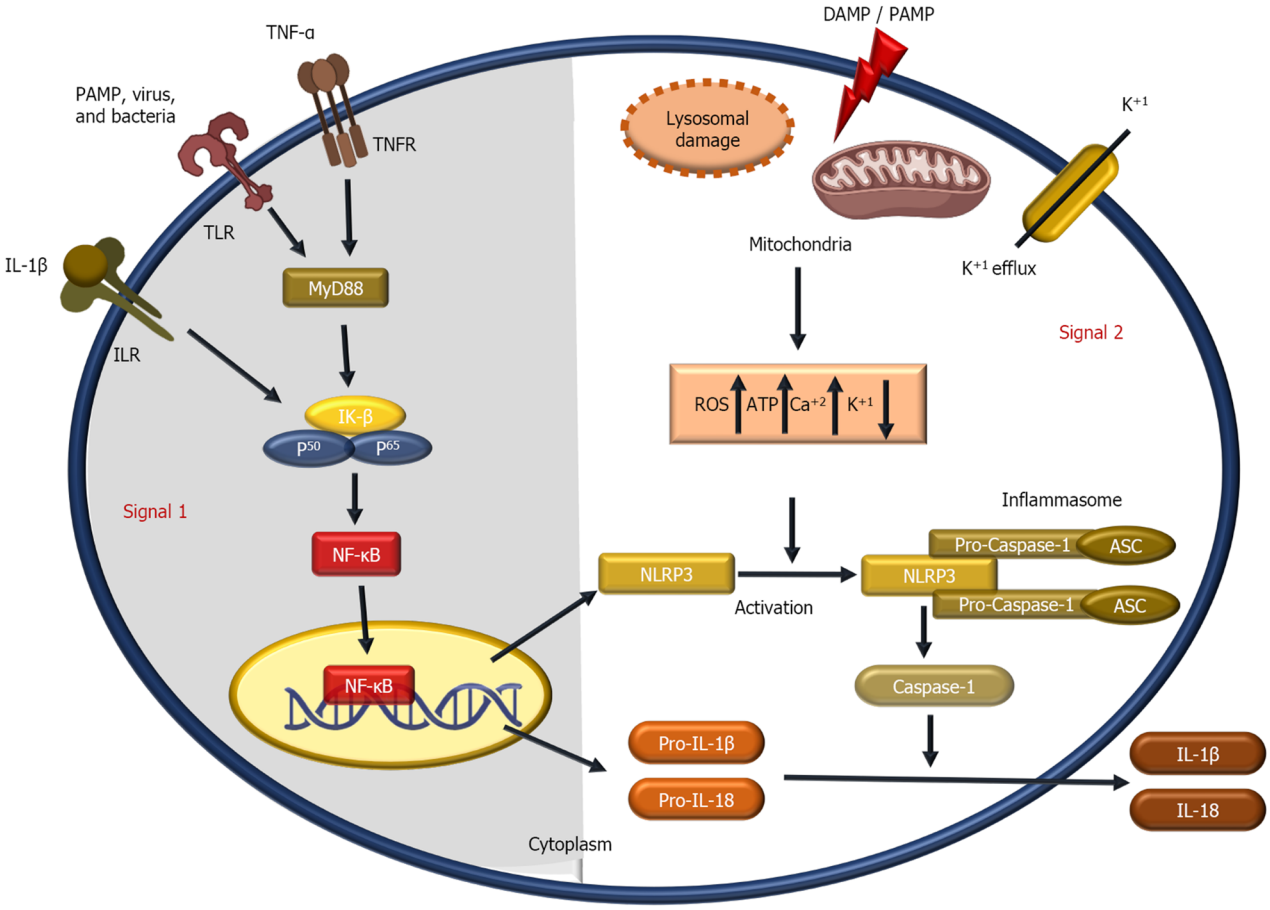
Ca^{2+} is necessary for NLRP3 inflammasome activation by interacting with inositol 1,4,5-trisphosphate, a byproduct of phosphatidylinositol 4,5-bisphosphate hydrolysis catalyzed by phospholipase C, and the inositol 1,4,5-trisphosphate receptor on the endoplasmic reticulum, which promotes Ca^{2+} mobilization and NLRP3 inflammasome activation[60]. The NLRP3 inflammasome is activated by excessive ER Ca^{2+} release associated with mitochondrial damage and results in ROS production and an excess of Ca^{2+}[74-76]. Additionally, K^+ efflux and a reduction in intracellular K^+ are upstream events in NLRP3 activation [59,77]. The NLRP3 inflammasome also needs K^+ efflux for NLRP3 formation, according to a recently discovered component called NIMA-related kinase 7 (NEK7), which may directly bind to NLRP3 protein[78,79]. Various substances can increase ROS generation and induce NLRP3 activation without requiring K^+ efflux[80,81]. Na^+ influx-induced NLRP3 activation also relies on K^+ efflux[59]. Reducing the concentration of extracellular Cl^- activates caspase-1 and the generation of IL-β1[82]. An anion channel called the intracellular chloride channel has the potential to activate the volume-regulated anion channel. It is intriguing since the K^+ efflux-mitochondrial ROS axis was shown to be a downstream event of chloride intracellular channel-dependent chloride efflux. Intracellular chloride channel-mediated chloride efflux can encourage NEK7-NLRP3 association and subsequent ASC oligomerization[83].

ROS are one of the earliest identified triggers to engage the NLRP3 inflammasome. Lysosomal NADPH oxidase was once believed to be the source of ROS formation, even though mitochondria are the primary site of ROS production[59,84]. According to several studies, most NLRP3 inflammasome agonists have been shown to produce mitochondrial ROS in various cell types[63,85-88]. NADPH oxidase 4 (NOX4) has also been shown to influence carnitine palmitoyl transferase 1A and promote fatty acid oxidation, which aids NLRP3 activation[89].

Lysosomal destabilization contributes to NLRP3 activation in both phases (signal 2) and the priming step (signal 1); in palmitate-induced NLRP3 activation. By controlling the stability of the IL-1 mRNA, lysosomal calcium signaling controls the production of pro-IL-1β (signal 1), whereas cathepsin B, a lysosomal protease, contributes to the NLRP3 activation[90]. It has been hypothesized that acidic conditions are necessary for monosodium urate crystals to activate the inflammasome within lysosomes as the significant Na^+ release raises the cellular osmolality and water influx and lowers intracellular K^+ concentration[91].

POST-TRANSCRIPTIONAL MODIFICATIONS OF NLRP3

Protein folding, localization, and functional activity are all controlled by post-translational modifications. Numerous post-translational modifications have been shown to affect innate immunity by affecting immune cells' activation, survival, differentiation, and migration[92]. NLRP3 is ubiquitylated in dormant macrophages and deubiquitylated following activation and priming[93]. For the NLRP3 inflammasome to activate, the linear ubiquitin assembly complex must selectively ubiquitylate NLRP3 and ASC[94]. TLR priming enhances NLRP3 self-association and activation by causing Jun N-terminal kinase 1 to phosphorylate NLRP3[95]. The mechanism of NLRP3 inflammasome activation is outlined in Figure 2.



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Figure 2 The mechanism of nod-like receptor protein-3 inflammasome activation. Nod-like receptor protein-3 (NLRP3) inflammasome activation occurs through two signals, (signal 1) toll-like receptor stimulation by pathogen associated molecular pattern (PAMP), bacteria and viruses, tumour necrosis factor receptor stimulation by TNF-α, and interleukin receptor (ILR) stimulation by IL-1β leads to activation of nuclear factor-kappa B (NF-Kb) *via* upregulation of MyD88, and Iκ-β, P50, and P65 complex. Pro-IL-1β, pro-IL-18, and inactive NLRP3 expression were all increased by the translocation of activated NF-κB into the nucleus. (Signal 2) Increased intracellular reactive oxygen species, adenosine triphosphate, and Ca^{+} levels are caused by damage associated molecular pattern and PAMP-induced lysosomal damage, and mitochondrial dysfunction, while K^{+} efflux lowered intracellular K^{+} level. The previous intracellular events lead to activates NLRP3 to inflammasome that promotes caspase-1 which begin to convert pro-IL-1β and pro-IL-18 into IL-1β and IL-18. IL-(1β): Interleukin-1 beta; DAMP: Damage associated molecular pattern; PAMP: Pathogen associated molecular pattern; TNF-α: Tumour necrosis factor-alpha; ILR: Interleukin receptor; TLR: Toll-like receptor; TNFR: Tumour necrosis factor receptor; MyD88: Myeloid differentiation primary response 88; IK-β: Inhibitor *kappa*-beta; P50: Nuclear factor-kappa B P50 subunit; P65: Nuclear factor-kappa B P65 subunit; ROS: Reactive oxygen species; ATP: Adenosine triphosphate; Ca+: Calcium ion; K+: Potassium ion; NF-Kb: Nuclear factor-kappa B; NLRP3: Nod-like receptor protein-3; ASC: Apoptosis-associated speck-like protein.

THERAPEUTIC AVENUES FOR ULCERATIVE COLITIS TARGETED NLRP3 INFLAMMASOME SIGNAL

Phytochemicals and plant extracts

Naringin: Naringin is a flavonoid glycoside, rich in citrus fruits such as grapefruit and orange[96,97]. Naringin protects mice from obesity, azoxymethane/dextran sodium sulfate (DSS)-induced carcinogenesis, and acetic acid (AA)-induced colitis[98-100]. In 2018, Cao *et al*[101] revealed that naringin protects mice from DSS-induced UC owing to its NLRP3 inflammasome and mitogen-activated protein kinase (MAPK) inhibitory effects. Furthermore, it upregulated peroxisome proliferator-activated receptor γ (PPARγ) while suppressing NF-κB activation.

Chlorogenic acid: Chlorogenic acid (CGA) is a phenolic acid found in foods and many plants[102,103]. Zeng *et al*[104] reported that CGA prevented DSS-induced mice colitis by down-regulating miR-155 expression and suppressing NLRP3 and NF-κB in macrophages. CGA downregulates caspase-1, pro-cleaved-IL-1β, and pro-cleaved-IL-18 in mice with colitis. In line with this study, CGA ameliorates NLRP3 inflammasome in different models, such as CCl_4-induced acute liver injury[105] and lipopolysaccharide (LPS)-induced mice with acute lung injury[106].

1, 25-dihydroxy vitamin D3: Vitamin D (Vit D3) is a fat-soluble vitamin that maintains calcium and phosphorus homeostasis[107]. Significantly, Vit D3 ameliorates the NLRP3 inflammasome in variant mouse models like periodontitis[108], diabetes-induced retinal vascular damage[109], nitrogen mustard-induced cutaneous inflammation[110], hyperosmotic stress-induced injury[111], cisplatin-induced acute renal injury[112], and allergy inhibition[113]. Cao *et al*[114] reported that it suppresses NLRP3 inflammasome activation, NLRP3-mediated ASC oligomerization, caspase-1 activation, and IL-1β production. Also, NLRP3 binding to NEK7 was inhibited in DSS-induced UC.

Ginsenosides: Ginsenosides are the most significant phytochemical of ginseng obtained from the *Panax* species family *Araliaceae*[115,116]. Ginsenoside Rk3 (Rk3) is a tetracyclic triterpenoid reportedly found in Radix notoginseng herbs[117]. Tian *et al*[118] revealed that Rk3 protects against DSS-induced UC by suppressing NLRP3 expression. Rk3 administration significantly attenuated myeloperoxidase (MPO), inducible nitric oxide synthase activities, and downregulated NLRP3, ASC, and caspase-1 expression. Ginsenoside Rd (Rd) is another ginsenoside that inhibits NLRP3 in UC[119]. Rd suppresses NLRP3 activation and decreases caspase-1 production and IL-1β levels in DSS-induced UC. Also, Rd inhibits MPO and inducible nitric oxide synthase activities while increasing antioxidant glutathione content [120].

Phloretin: Phloretin (PHL) is a flavonoid found in fruit, leaves, and roots of the apple tree *Malus domestica*[121,122]. Zhang *et al*[123] demonstrated that PHL suppressed DSS-induced NLRP3 inflammasome activations and regulated the NF-κB, TLR4, and PPARγ pathways. Moreover, PHL attenuated oxidative stress and regulated the expression of ZO-1 and occludin. In the same context, Wu *et al*[124] found that PHL ameliorated UC by suppressing NF-κB and NLRP3 activation. Additionally, it decreases cytokines and oxidative injury while maintaining intestinal barrier integrity.

Cinnamaldehyde: Cinnamaldehyde (CA) is the principal constituent of the bark of *Cinnamomum cassia* and *C. verum*[125]. CA suppresses NLRP3 in several models like rheumatoid arthritis[126], endotoxin-poisoned mice[127], cardiac ischemia-reperfusion[128], fructose-induced cardiac inflammation and fibrosis[129], and LPS-induced renal inflammation[130]. CA mitigates DSS-induced colitis by suppressing NLRP3 activation and miR-21, miR-155, IL-1β, and IL-6 levels. Furthermore, the suppression of NLRP3, miR-21, and miR-155 was observed in CA-treated, LPS-stimulated RAW264.7 cells. The production ROS and protein kinase B/mammalian target of rapamycin phosphorylation was also reduced[131].

Palmatine: Palmatine is an isoquinoline alkaloid found in traditional Chinese medicine (TCM), including *Fibraurea spp.*, *Corydalis spp.*, *Berberis spp.*, and *Phellodendron spp*[132-134]. Palmatine suppresses NLRP3 inflammasomes in different models, like hyperuricemia-induced kidney injury[135] and monosodium uric acid-induced gouty arthritis[136]. Mai *et al*[137] revealed that palmatine attenuates DSS-induced colitis in mice by suppressing NLRP3 inflammasomes and decreasing the colonic levels of MPO, IL-1β, and TNF-α. In 2022, Cheng and co-workers recently suggested that 8-oxypalmatine exerted an appreciable protective effect on DSS-induced colitis, suppressing the NLRP3 inflammasome and activating the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway[138].

L-fucose: L-fucose, a naturally safe monosaccharide found in animals, is often utilized as a food ingredient[139,140]. L-fucose attenuates colitis by suppressing NLRP3 inflammasome and NF-κB activation, decreasing pro-inflammatory cytokines, and reducing neutrophil and macrophage infiltration[141].

Genistein: Genistein (Gen) is a significant isoflavone in soy[142]. Gen suppresses the NLRP3 inflammasome in macrophages by activating the G protein–coupled receptor 5-cAMP signal in DSS-induced UC mice. Additionally, the Gen administration boosted intracellular cAMP levels and decreased caspase-1 and IL-1β levels[143]. In agreement with this study, Gen suppressed the NLRP3 inflammasome in different models, such as cerebral ischemia in mice[144], H_2O_2-induced senescence of human umbilical vein endothelial cells[145], hippocampal neurons in aging rat brain tissue[146], and alloxan-induced diabetes in mice[147].

Sinapic acid: Sinapic acid (SA) is a phytochemical abundant in various food plants, including spices, citrus and berry fruits, cereals, and others[148-150]. SA was shown to have potential therapeutic efficacy in inhibiting NLRP3-associated inflammatory disorders[151]. In conjunction with that, SA upregulated tight junction protein-1 (ZO-1) and claudin-1 in DSS-induced UC, suppressing the NLRP3 activation and attenuating intestinal permeability. Additionally, SA attenuates oxidative injury by boosting antioxidants superoxide dismutase, glutathione peroxidase, and catalase activity while lowering MPO and pro-inflammatory cytokine mRNA levels in circulation and colonic tissue[152].

Terpinen-4-ol: Terpinen-4-ol (TER) is a primary ingredient in *Zanthoxylum bungeanum* Maxim's essential oil[153]. Zhang *et al*[154] investigated whether the protective effect of TER may be substantially related to its suppression of NLRP3 activation in DSS-induced colitis. TER balanced the amounts of *Lactobacillus* and *Escherichia coli* while lowering the plasmatic LPS content. Additionally, TER inhibited the

breakdown of the colon epithelial barrier by controlling the expression of ZO-1 and occluding and decreasing IL-1β production.

Apigenin: Apigenin (API) is a flavone found in many vascular plants' leaves, vegetables, stems, and fruits[155]. According to Márquez-Flores *et al*[156], API anti-inflammatory activity was attributed to a suppression of the NLRP3 inflammasome by lowering levels of pro-inflammatory cytokines and oxidative stress as a result of controlling the activity of cleaved caspase-1 and caspase-11 enzymes. In agreement with this study, API suppresses NLRP3 inflammasome activation in high-fat diet-induced non-alcoholic fatty liver disease[157], palmitic acid-induced liver injury[158], arteriosclerosis[159], human umbilical vein endothelial cells inflammation[160], depression in chronic unpredictable mild stress rats[161], and doxorubicin-induced renal injury[162].

Rosmarinic acid: Rosmarinic acid (RA) is a natural polyphenol found in plants from the *Labiatae* family [163]. RA down-regulates the NRLP3-inflammasome in neuroinflammation[164], acetaminophen-induced liver injury[165], and nicotine-induced atherosclerosis[166]. In 2021, Marinho *et al*[167] demonstrated that RA-loaded nanovesicles enhanced Nrf2 and heme oxygenase-1 expression while decreasing NLRP3 inflammasome, ASC, and caspase-1 protein expression and IL-1β levels. RA also reduces TNF-α and MPO activity.

Lonicerin: A flavonoid glycoside, lonicerin, is extracted from the plant *Lonicera japonica* Thunb. It treats infectious and inflammatory diseases[168,169]. Numerous investigations have shown that lonicerin is among the main ingredients with anti-inflammatory and immunomodulatory properties[170]. Lv *et al* [165] suggested that lonicerin reduces colitis through NLRP3-ASC-pro-caspase-1 complex assembly. Lonicerin has a therapeutic impact on intestinal inflammation by directly binding to the histone methyltransferase enhancer of zeste homolog 2, which promotes autophagy and speeds up the destruction of NLRP3 by autolysosomes.

Magnesium lithospermate B: Magnesium lithospermate B (MLB) is the primary ingredient of *Salviae miltiorrhizae*[171]. It has been reported that MLB treats acute colitis[172,173]. Jiang *et al*[174] found that MLB might treat acute and chronic colitis by downregulating NLRP3, ASC, and caspase-1 levels.

Fumigaclavine C: Fumigaclavine C is an indole alkaloid obtained from endophytic *Aspergillus terreus* and *Rhizophora stylosa*[175,176]. According to Guo *et al*[177], fumigaclavine C attenuates DSS-induced colitis by decreasing pro-inflammatory cytokines TNF-α, IL-1β, and IL-17A, mediated by suppressing NLRP3 inflammasome and caspase-1 activation.

Nigeglanine: Nigeglanine is a phytochemical obtained from *Nigella sativa* oil[178]. According to a study conducted in 2019 by Gao *et al*[179], nigeglanine inhibits colon epithelial cell pyroptosis by suppressing the NLRP3 inflammasome, NF-κB, and MAPK in DSS-induced colitis in mice. Surprisingly, nigeglanine supplementation enhanced ZO-1 and occludin protein levels, indicating that nigeglanine protects barrier integrity.

Mycophenolate mofetil: Mycophenolate mofetil (MMF) is a fermentation byproduct of *Penicillium stoloniferum*[180-182]. MMF is an immunosuppressant commonly used to treat systemic lupus erythematosus, lupus nephritis, and other autoimmune disorders[183]. Serrya *et al*[184] observed that MMF had coloprotective effects against AA-induced UC by suppressing the NLRP3 inflammasome and consequent release of IL-1β, IL-18, and INF-γ. In colon tissues, MMF considerably reduced oxidative stress and boosted antioxidants glutathione, catalase, and superoxide dismutase levels as well as total antioxidant capacity mediated by upregulating Nrf-2.

Evodiamine: Evodiamine (EVO), a significant alkaloidal substance discovered from *Evodia rutaecarpa*, has long been used in TCM to treat various infection-related diseases, such as diarrhea, beriberi, and oral ulcers[185,186]. Additionally, EVO effectively treats nausea and abdominal discomfort[187]. Furthermore, EVO inhibits NLRP3 inflammasome activation, indicating the application of EVO in treating IBD[188] and enhancing innate immunity against bacterial infection[189]. In DSS-induced UC mice, EVO reduced inflammation by controlling the NF-κB signal and NLRP3 inflammasome, which reduced the production of TNF-α, IL-1β, and IL-6 and altered the expression of ZO-1 and occludin[190]. Similarly, Ding *et al*[188] showed that EVO inhibits NLRP3 inflammasome activation by enhancing autophagosome degradation and inhibiting the NF-κB pathway.

Brusatol: Brusatol is a major quassinoid of *Brucea javanica* used in treating inflammatory disorders, particularly intestinal inflammation, such as colitis and dysentery[191]. Zhou *et al*[192] revealed that the anti-UC activity of brusatol is closely related to the suppression of NF-κB and NLRP3 signals and the activation of Nrf2 expression-mediated anti-oxidative effects. The TNF-α, pro- IL-1β, IL-18, prostaglandin E2, and nitric oxide levels were significantly reduced by brusatol in LPS-stimulated macrophages.

Bergenin: Bergenin, a C-glycoside produced from 4-O-methyl gallic acid, is one of the primary active components of plants in the genus *Peltophorum*[193]. Bergenin reduces palmitic acid-induced pancreatic injury by suppressing NLRP3 activation[194]. Likewise, Lopes de Oliveira *et al*[195] showed that bergenin attenuates 2,4,6-trinitrobenzene sulfonic acid-induced acute colitis in rats. Bergenin decreased inflammatory cytokines levels mediated by suppressing phosphorated signal transducer and activator of transcription 3 (STAT3), NF-κB, and NLRP3/ASC inflammasome signals.

Wedelolactone: Wedelolactone is a coumarin obtained from plants found in *Wedelia chinensis*[196,197]. Wei *et al*[198] revealed that wedelolactone administration significantly suppressed NLRP3 and caspase-1 activation to reduce IL-1β production in DSS-induced UC. Wedelolactone also successfully maintains intestinal barrier function by activating AMP-activated protein kinase (AMPK).

Walnut oil: Walnut oil is commonly recommended as a nutritious, premium food oil and is used extensively in TCM[199]. Walnut oil has anti-inflammatory and antioxidant effects[200-202]. Miao *et al*[203] explored how walnut oil inhibits the NLRP3 inflammasome activation and modifies gut microbiota in DSS-induced colitis in mice. Walnut oil enhanced the levels of short-chain fatty acids and blocked apoptosis while reducing ROS generation and pro-inflammatory cytokines release by suppressing NLRP3, ASC, and caspase-1 activation.

Nigakinone: Nigakinone is the principal active ingredient of *Ramulus et Folium Picrasmae*, and it is one of the TCM substances frequently used to treat diarrhea, colitis, and dysentery[204]. In DSS-induced colitis, nigakinone alleviated symptoms by regulating the farnesoid X receptor/NLRP3 pathway[205].

Oroxindin: Oroxindin is a natural bioflavonoid that was identified in Huang Qin. Oroxindin has been shown in several studies to have a variety of bioactivities, including anti-inflammatory, antioxidant, and anticancer effects[206-208]. According to Liu *et al*[209], oroxindin reduces inflammatory responses by suppressing NLRP3 inflammasome activation and reducing colonic IL-1β and IL-18 Levels. Oroxindin modulated the production of thioredoxin interacting protein (TXNIP), reducing LPS-induced NLRP3 inflammasome and caspase-1 activation in cultured macrophages, hence decreasing TXNIP-dependent NF-κB activation.

Schisandrin B: Schisandrin B is the most significant component in *Schisandra chinensis*[210]. Schisandrin B suppresses NLRP3 inflammasome activation in LPS-induced airway inflammation and airway remodeling[211] and propionibacterium acnes-induced pyroptosis[212]. In 2021, Zhang *et al*[213] demonstrated schisandrin B reduces NLRP3 inflammasome activation mediated by IL-1β release in intestinal epithelial cells of a colitis model.

Trifolirhizin: Trifolirhizin is a pterocarpan identified from *Trifolium pratense* and *Sophora flavescens*[214,215]. Trifolirhizin treatment successfully controlled the balance of T helper/T regulatory (Th17/Treg) cells and inflammation in DSS-induced UC mice by suppressing NLRP3 activation by TXNIP. Trifolirhizin has also been shown to control the AMPK-TXNIP pathway[216].

Sanguinarine: Sanguinarine, benzylisoquinoline alkaloids, is obtained from *Sanguinaria canadensis* and *Fumaria*[217]. According to Li *et al*[218], sanguinarine showed a potential therapeutic impact on DSS-induced UC mice by suppressing NLRP3-(Caspase-1)/IL-1β pathway and enhancing gut microbial dysbiosis. Additionally, sanguinarine decreased the expression of NLRP3 and the activation of caspase-1 and IL-1β in THP-1 cells induced by LPS[218].

Geniposide: The primary active ingredient of *Gardenia jasminoides* is geniposide, an iridoid glycoside extracted from the *Gardenia* used in TCM[219]. Geniposide was shown by Pu *et al*[220] to reduce macrophage differentiation in mice with acute colitis induced by DSS by inhibiting the NLRP3. In addition, geniposide augmented AMPK/Sirt1 signal and inhibited the NLRP3 inflammasome in LPS-induced BMDM cell or RAW264.7 cell cultures. In line with this study, geniposide suppressed the activation of the NLRP3 inflammasome in different *in vivo* and *in vitro* models, such as myocardial ischemia/reperfusion injury[221], sepsis-induced myocardial dysfunction[222], and cholestatic liver inflammatory injury[223].

Sinomenine hydrochloride: Sinomenine is a pure alkaloid derived from the plant *Sinomenium acutum* of the *Menispermaceae* family[224]. According to Zhou *et al*[225], sinomenine may reduce the symptoms of experimental colitis by altering the production of the gut microbiota and reducing the activity of the NLRP3 inflammasome and pro-inflammatory mediators levels in mice. In line with this study, sinomenine inhibits the NLRP3 inflammasome, which has a favorable effect on cartilage degradation [226], autoimmune encephalomyelitis[227], ischemic stroke[228], and others.

Picroside II: Picroside II is a kind of iridoid chemical that is used in TMC. There are now three known iridoids in *Picrorhiza scrophulariiflora* *pennell*[229]. Picroside II possesses hepatoprotective, immune-regulating, anti-inflammatory, and antioxidant properties[230-232]. Yao *et al*[233] demonstrated that picroside II alleviated DDS-induced UC by significantly suppressing NLRP3 inflammasomes and

inflammatory components *in vivo* induced by DDS.

Hydroxytyrosol: Hydroxytyrosol is a prominent and typical phenolic component in olive oil and leaves [234]. Miao[235] demonstrated that hydroxytyrosol supplementation has a coloprotective effect on DSS-induced UC by suppressing NLRP3, caspase-1, and ASC expression levels and downregulating IL-18 and IL-1β levels. Additionally, hydroxytyrosol modifies the *in vivo* gut microbiota while boosting colonic antioxidant functionality.

Trans-10-hydroxy-2-decenoic acid: The most prevalent fatty acid and main lipid in royal jelly is 10-hydroxy-2-decenoic acid, which also has antibacterial[236], antioxidant[236], immunomodulatory[237], anti-inflammatory[238] properties, and others. According to Huang *et al*[239], trans-10-hydroxy-2-decenoic acid improves colonic barrier function, decreases colonic TXNIP, ASC, caspase-1, GSDMD, IL-1β, and IL-18 Levels, and regulates the NLRP3 inflammasome-mediated pyroptosis to treat DSS-induced colitis.

PLANT EXTRACT

Canna x generalis

Canna x generalis (CG) LH Bailey is a hybrid of two closely related species that belongs to the *Canna* genus[240]. With its enormous leaves and gorgeous blooms, CG may grow without special care and can adapt to various soil conditions[241,242]. CG exhibits anti-inflammatory[243] and antioxidant[244] activities. CG extract exhibits a protective effect against DSS-induced UC mice by suppressing colonic activation of NLRP3 and NF-κB/TLR4 signals. CG extract reduces pro-inflammatory mediators, oxidative stress, and the inflammatory cascade in colonic tissues. Additionally, CG extract downregulates caspase-1, ASC, TLR4, and caspase-3 expressions[245].

Kuijieling decoction: An empirical formula known as Kuijieling decoction (KJL) has been introduced in clinical conditions for several years and is effective in treating UC[246,247]. KJL showed anti-inflammatory and antioxidant bioactivities[248,249]. KJL effectively attenuates UC symptoms in the UC rat model[250]. Jie *et al*[251] revealed that KJL suspension attenuates UC by decreasing NLRP3, caspase 1, and pro-inflammatory cytokines levels. They also showed that in DSS-induced UC mice, KJL downregulates NLRP3, caspase-1, GSDMD-N, IL-1β, and IL-18. Also, the expression levels of ASC, caspase-1, IL-1β, IL-18, and Mir-223 were downregulated in colon tissue.

Huaier: For more than 1600 years, *Trametes robiniophila Murr* (Huaier), a sandy beige fungus that grows on tree trunks, has been extensively employed in TCM[252-254]. Wang *et al*[255] showed that Huaier has anti-inflammatory activity in DSS-induced murine colitis in mice by downregulating NLRP3 expression and preventing NLRP3 inflammasome activation-induced IL-1β production and caspase-1 cleavage.

Shaoyao decoction: Shaoyao decoction (SYD) is a TCM that has demonstrated that it has anti-inflammatory[256] and anticancer[257] effects, among others. In addition, a recent study revealed that SYD had been successfully utilized to treat IBD and other disorders linked to the damp-heat syndrome in the intestines[258]. Wei *et al*[259] reported the protective effects of SYD on DSS-induced UC and pyroptosis by suppressing the NLRP3 and NF-κB/P38 signals. Moreover, SYD supplementation decreases caspase-1 activity and the release of ASC and GSDMD, preventing the formation of NLRP3 and protecting the intestinal barrier integrity.

Smilax china L.

Smilax china L. is one of the TCM that is effective in reducing inflammation[260], nociception[261], and cancer[262]. *S. china L.* polysaccharide (also known as SCLP) was isolated from the rhizome of *S. china* L. and is a TCM used to treat inflammatory disorders[263]. SCLP treats UC, according to Pan *et al*[264], by inhibiting the galectin-3 expression and its connection with NLRP3 activation and IL-1β production, which in turn decreases the release of inflammatory mediators in the DSS mice model.

Dendrobium officinale

Dendrobium officinale Kimura et Migo is the second-biggest genus of *Orchidaceae*[265,266]. Polysaccharides, the main active constituents in *D. officinale*, have received much interest due to their numerous biological activities[267-269]. Moreover, a prior investigation showed that *D. officinale* polysaccharides (DOPS) could successfully alleviate DSS-induced colitis in mice[270]. In 2018, Liang *et al*[270] found that DOPS has a notable therapeutic effect in treating DSS-induced acute UC mice. They also examined whether DOPS administration might significantly reduce the activation of the NLRP3 inflammasome and the β-arrestin1 signaling pathways. DOPS significantly decreases colonic pathological damage, relieves clinical signs and symptoms, and restores the pro- and anti-inflammatory cytokines balances.

Schisandra chinensis

As a result of its various pharmacological properties, the fruit of *Schisandra chinensis*, a member of the *Magnoliaceae* family, has long been utilized as an herbal remedy in TCM[271,272]. These fruits were often utilized as wholesome meals and pharmaceuticals for several chronic diseases, such as cancer, liver injuries, and gastrointestinal disorders[210,273]. *S. chinensis* mitigates ferroptosis and NLRP3 inflammasome-mediated pyroptosis in diabetic nephrosis[274]. Recently, Bian *et al*[275] found that *S. chinensis* extract can reduce the symptoms of DSS-induced colitis by TLR4/NF-κB and NLRP3 inflammasome suppression. Additionally, *S. chinensis* extract might correct the gut microbiota imbalance brought on by UC while maintaining gut barrier function by raising ZO-1 and occludin levels.

Tripterygium wilfordii

Tripterygium wilfordii Hook. f., commonly referred to as Lei Gong Teng, is a TCM herb frequently used to treat autoimmune and inflammatory diseases[276-278]. The root bark of *T. wilfordii* demonstrated pharmacological actions against inflammation[279], autoimmune disorders[280], Crohn's disease[281], liver cancer[282], and others. *T. wilfordii* polycoride is the primary active ingredient of *T. wilfordii*[283]. Fangxiao *et al*[284] reported the anti-inflammatory effects of *T. wilfordii* polycoride on 2,4,6-trinitrobenzenesulfonic acid-induced colitis by downregulating the NOXs-ROS-NLRP3 signal. *T. wilfordii* polycoride decreased ROS production, and NOXs' activity was mediated by downregulating colonic NLRP3, ASC, and caspase-1.

Rubia cordifolia L.

Rubia cordifolia L. is a perennial climbing vine from the Rubiaceae family[285,286]. Its aerial portion possesses a range of bioactivities, including anti-inflammatory[287], antioxidant[288,289], and others. Recent findings by Qin *et al*[290] showed that using *R. cordifolia* extract inhibits the NLRP3 inflammasome and IL-6/Jak-2/STAT3 signal activation in DSS-induced UC. *R. cordifolia* extract improves the symptoms, diminishes colonic mucosal injury and macrophage infiltration, inhibits the production of inflammatory cytokines, and lowers mortality.

Mulberry fruit extract

Mulberry fruit extract (MFE) is obtained from the fully ripened fruits of *Morus macroura*[291,292]. Salama *et al*[293] recently reported the involvement of NLRP3, miRNA-223, and the TNF-α/NF-κB pathway in the coloprotective effects of MFE against AA-induced UC in rats. MFE also reduces levels of TNFR1, NLRP3, NF-κB p65, TNF-α, IL-1β, IL-18, and caspase-1, while increases miRNA-223 expression.

PROBIOTICS

Akkermansia muciniphila

Akkermansia muciniphila, a Gram-negative and anaerobes bacterium, is a member of the *Verrucomicrobia phylum*, which has been discovered to be common in the human gut[294-296]. According to investigations, *A. muciniphila* may protect against major diseases like atherosclerosis[297], amyotrophic lateral sclerosis[298], and immune-mediated liver damage[299]. Additionally, it was reported that *A. muciniphila* improves chronic colitis[300]. Recently, Qu *et al*[301] revealed that oral treatment of *A. muciniphila* strain BAA-835 effectively reduces the signs and symptoms of DSS-induced acute colitis dependent on NLRP3 activation. The expression of NLRP3, caspase-1, and IL-1β was elevated in mouse macrophage cells in *A. muciniphila*-treated animals, as well as pro-inflammatory cytokines such as TNF-α, IL-6, and monocyte chemoattractant protein 1 (MCP-1).

Saccharomyces cerevisiae

A facultative anaerobic fungus called *Saccharomyces cerevisiae* has been extensively employed in medicine to create modified carriers and oral vaccinations[302,303]. According to a study by Sun *et al* [304], modified *S. cerevisiae* reduced the severity of DSS-induced colitis in mice by inhibiting macrophage pyroptosis and regulating the intestinal microbiota. Lactic acid-produced *S. cerevisiae* modulated macrophage polarisation, prevented the production of pro-inflammatory cytokines *in vivo* and *in vitro*, and decreased NLRP3 inflammasome and caspase-1 levels.

Lactobacillus acidophilus

Lactobacillus acidophilus is one of the most common commercial species of lactic acid bacteria, found in various dairy products and nutritional supplements with probiotic indications[305]. According to studies, *L. acidophilus* may have immunomodulatory, anti-inflammatory, and antioxidant[306-308] activities. Furthermore, it improves the intestinal epithelial barrier function[309,310]. Additionally, it was demonstrated to be efficient in preventing colitis caused by *Citrobacter rodentium*[309] and relieving DSS-induced colitis[311]. According to Li *et al*[312], *L. acidophilus* attenuates UC in rats by inhibiting the NLRP3 inflammasome pathway, increasing the short-chain fatty acids level, and promoting autophagy.

Grape seed proanthocyanidin

Grape seed proanthocyanidin extract (GSPE) is a grape seed extract that contains catechin, epicatechin gallate, and epigallocatechin[313,314]. According to Sheng *et al*[315], GSPE supplementation reduces inflammatory cytokines and oxidative stress, maintains the intestinal barrier, and enhances the microbiome in DSS-induced colitis. GSPE decreases the NLRP3 inflammasome and increases ZO-1, occludin, and claudin-1 levels. Furthermore, GSPE treatment of colon tissues resulted in a considerable decrease in TNF-α and IL-1β levels.

Brilliant blue G

Brilliant blue G (BBG) is a triarylmethane dye with a modest hydrophilicity that has been proven to stain the inner limiting membrane selectively[316]. In 2021, Saber *et al*[317] revealed that the BBG/OLT1177 combination produced complementary effects and significantly alleviated DSS-induced UC by downregulating NLRP3, caspase-1, IL-1β, and IL-18 levels. In addition to reducing purinergic receptor (P2X7R) and oxidative stress levels, BBG treatment downregulated myeloid differentiation primary response 88, NF-κB, IL-6, TNF-α, the recruitment of the NLRP3 inflammasome, and the consequent activation of caspase-1, IL-1β, and IL-18.

Forsythia suspensa extract

Forsythia suspensa (Thunb.) Vahl is well-known in TCM[318]. Chao *et al*[319] showed that *Forsythia suspensa* extract effectively reduces metabolic dysfunction and DSS-induced UC damage by suppressing the NLRP3 pathway and activating Nrf2.

Kui jie tong

Kui jie tong is a herbal extract that effectively treats UC[320]. Xue *et al*[321] reported that Kui jie tong mitigates UC by downregulating NLRP3 and caspase-1 as well as serum IL-1β, IL-18, and IL-33 and enhancing intestinal microbiota.

Jianpi Qingchang decoction

Jianpi Qingchang decoction, a TCM prescription, is composed of nine Chinese herbs and effectively attenuates moderate or beginning cases of UC[322,323]. Zhang *et al*[324] stated that Jianpi Qingchang decoction has a protective function by preventing DSS-induced NLRP3 inflammasome activation. Jianpi Qingchang decoction decreases inflammatory cytokine release.

Bovine milk

Bioactive extracellular vesicles are present in the milk of all mammalian species[325,326]. Bovine milk exosomes have been shown to support intestinal cell proliferation, boost the number of goblet cells and mucin synthesis, inhibit bacterial growth, and support the intestinal microbiota[327-329]. According to the Tong *et al*[330] study, milk-derived extracellular vesicles regulate intestinal immunological homeostasis by suppressing TLR4- NF-κB and NLRP3 signaling pathways, re-establishing the balance of Treg/Th17 cells, and altering the gut microbiota. In a mouse UC model, milk-derived extracellular vesicles were proven to have a role in the control of immunological and inflammatory pathways, which decreased intestinal epithelium disruption, blocked the infiltration of inflammatory cells, and reduced tissue fibrosis[330].

SYNTHETIC DRUGS

Mirtazapine

Mirtazapine is a well-known antidepressant medication used to treat depression, anxiety, and sleep disorders[331]. In addition, mirtazapine reduces inflammation in diabetic rat kidneys by inhibiting the NLRP3 inflammasome[332]. The same results showed that mirtazapine has a coloprotective effect against UC, which was mediated by suppressing NLRP3 and caspase-1 activation and restoring the antioxidant/oxidant balance in AA-induced UC in rats. Additionally, mirtazapine reduces the levels of NF-κB, TNF-α, IL-1β, and IL-18[333].

Paeoniflorin-6'-O-benzene sulfonate

Paeoniflorin-6′-O-benzene sulfonate is a new active monomer created by structurally altering paeoniflorin[334] and has anti-inflammatory and immunomodulatory characteristics[335,336]. According to Li *et al*[337], paeoniflorin-6′-O-benzene sulfonate has anti-colitis properties *via* suppressing TLR4-NF-κB and NLRP3 signals and G protein-coupled receptor kinase 2 translocations. Furthermore, paeoniflorin-6′-O-benzene sulfonate maintains intestinal barrier function in LPS-treated mice.

Dapagliflozin

Dapagliflozin (DPZ), a sodium-glucose cotransporter-2 inhibitor, is recommended in addition to routine medical treatment for managing type 2 diabetes mellitus that has not been effectively controlled[338]. Interestingly, DPZ modulates the NLRP3 inflammasome in different models, such as diabetic nephropathy[339], LPS-induced lung damage[340], and type 2 diabetes mellitus- induced cardiomyopathy[341]. El-Rous and colleagues reported that DPZ suppressed the activation stage (signal 2) of NLRP3 inflammasome activation and prevented the priming step (signal 1) of that activation in AA-induced UC through altering NF-κB/AMPK interaction and halting NLRP3/caspase-1 communication. Furthermore, DPZ increases the anti-inflammatory cytokine IL-10 and remarkably suppresses caspase-1 activity and IL-1β and IL-18 production[342].

Canagliflozin

Canagliflozin is a small-molecule hypoglycemic medication that lowers blood glucose levels by blocking the type 2 sodium-glucose cotransporter, which prevents the kidneys from reabsorbing glucose[343]. Nasr *et al*[344] reported that canagliflozin-loaded chitosan-hyaluronic acid microspheres significantly suppressed NF-κB and NLRP3 activation, resulting in a decrease in caspase-1 cleavage and the inhibition of several inflammatory cytokines, including IL-1β and IL-18, in AA-induced colitis.

Rosuvastatin

Rosuvastatin, an HMG-CoA inhibitor that is relatively new, has a safety and tolerability profile that is comparable to or better than the regularly prescribed dosages of other statins[345]. Rosuvastatin and *Lactobacillus* inhibit the NLRP3 inflammasome assembly, pro- IL-1β, pro-IL-18, and NF-κB, decreasing caspase-1 activity and IL-1β-driven pyroptotic activity in DSS in high-fat diet-induced colitis in rats [346].

MCC950

MCC950 is an IL-1β inhibitor and is known to be a potent inhibitor of the NLRP3 inflammasome[347]. MCC950 inhibits NLRP3 among inflammasomes[347,348] and blocks canonical, non-canonical, and alternative NLRP3 activation[80,347,349]. MCC950 showed promising therapeutic potential for inhibiting NLRP3 inflammasome in the atherosclerotic lesion[350], cholestatic liver, non-alcoholic steatohepatitis-induced liver fibrosis[351,352], LPS-induced lung inflammation[353], and others. In 2018, Perera *et al*[354] revealed that MCC950 effectively treats murine UC. MCC950 significantly suppresses NLRP3 inflammasome activation and decreases colonic cytokines, chemokines, and nitric oxide levels. Moreover, MCC950 suppresses caspase-1 activation in the colon and macrophage cells.

CONCLUSION

The incidence and prevalence of UC, a recurrent and remitting condition, are rising. The goal of treatment is to increase a patient's quality of life while achieving quick symptom alleviation and mucosal healing. There are still a lot of unsolved concerns despite the abundance of evidence pointing to genetic and host-related variables in UC. Numerous opportunities exist for further inquiry to improve our understanding of the pathogenesis of UC and uncover possible predictors of disease severity, responsiveness to medication, and novel therapeutic targets as the incidence and prevalence of UC around the world increase. The NLRP3 inflammasome plays a crucial role in the inflammation and immunological reaction by promoting caspase-1 activation and the release of IL-1β. Interestingly, phytochemicals, plant extracts, and synthetic drugs exhibit promising colon protective effects mediated by NLRP3 and caspase-1 activation suppression and, subsequently, the release of the pro-inflammatory cytokine IL-1β, which has a key role in UC-associated inflammation. However, future clinical studies are required to comprehend how NLRP3 inflammasome activation inhibition significantly treats and controls UC.

FOOTNOTES

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ORCID number: Fares E.M Ali 0000-0001-8341-7458; Osama M Ghogar 0000-0002-2003-1408.

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MINIREVIEWS

Immunotherapy for hepatocellular carcinoma: Current status and future perspectives

Deepa S Mandlik, Satish K Mandlik, Heena B Choudhary

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Deepa S Mandlik, Heena B Choudhary, Department of Pharmacology, BVDU, Poona College of Pharmacy, Pune 411038, Maharashtra, India

Satish K Mandlik, Department of Pharmaceutics, BVDU, Poona College of Pharmacy, Pune 411038, Maharashtra, India

Corresponding author: Deepa S Mandlik, PhD, Assistant Professor, Department of Pharmacology, BVDU, Poona College of Pharmacy, Kothrud, Pune 411038, Maharashtra, India. deepa.mandlik@bharatividyapeeth.edu

Abstract

Hepatocellular carcinoma (HCC) is one of the world's deadliest and fastest-growing tumors, with a poor prognosis. HCC develops in the context of chronic liver disease. Curative resection, surgery (liver transplantation), trans-arterial chemoembolization, radioembolization, radiofrequency ablation and chemotherapy are common treatment options for HCC, however, they will only assist a limited percentage of patients. Current treatments for advanced HCC are ineffective and aggravate the underlying liver condition. Despite promising preclinical and early-phase clinical trials for some drugs, existing systemic therapeutic methods for advanced tumor stages remain limited, underlining an unmet clinical need. In current years, cancer immunotherapy has made significant progress, opening up new treatment options for HCC. HCC, on the other hand, has a variety of causes and can affects the body's immune system *via* a variety of mechanisms. With the speedy advancement of synthetic biology and genetic engineering, a range of innovative immunotherapies, such as immune checkpoint inhibitors [anti-programmed cell death-1 (PD-1), anti-cytotoxic T lymphocyte antigen-4, and anti-PD ligand 1 cell death antibodies], therapeutic cancer vaccines, engineered cytokines, and adoptive cell therapy have all been used for the treatment of advanced HCC. In this review, we summarize the present clinical and preclinical landscape of immunotherapies in HCC, critically discuss recent clinical trial outcomes, and address future perspectives in the field of liver cancer.

Key Words: Hepatocellular carcinoma; Immunotherapy; Immune checkpoint inhibitors; Alpha-fetoprotein; Cancer vaccine; Combination therapies

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Core Tip: Hepatocellular carcinoma (HCC) is a multifaceted illness with multiple faces. It avoids early discovery, which provides the best chance of cure by resection/transplant, and systemic treatments are only of marginal efficacy at best, despite recent therapeutic advances. Current advances in immunotherapy and its combinations have altered the HCC treatment landscape, and clinical studies are continuing to pave the path forward. Immunotherapy increases survival rates and provides long-term cancer control in subsets of HCC patients while also minimizing side effects. Further research into immunotherapy in combination with current treatments for HCC in the early and intermediate stages may assist a greater spectrum of patients. Continued research into programmed cell death-1/ programmed cell death ligand 1, TMB, ctDNA, microsatellite stability, DNA mismatch repair, neutrophil/lymphocyte ratio, cytokines, and cellular peripheral immune response will hopefully identify the most reliable marker for selecting and sequencing systemic treatments to achieve the best outcome in HCC patients. Despite such significant treatment advances in HCC, numerous hurdles remain. The scientific community must figure out how to appropriately sequence these medicines for the best potential response, how to control toxicities, and how to develop indicators to monitor for response and relapse.

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INTRODUCTION

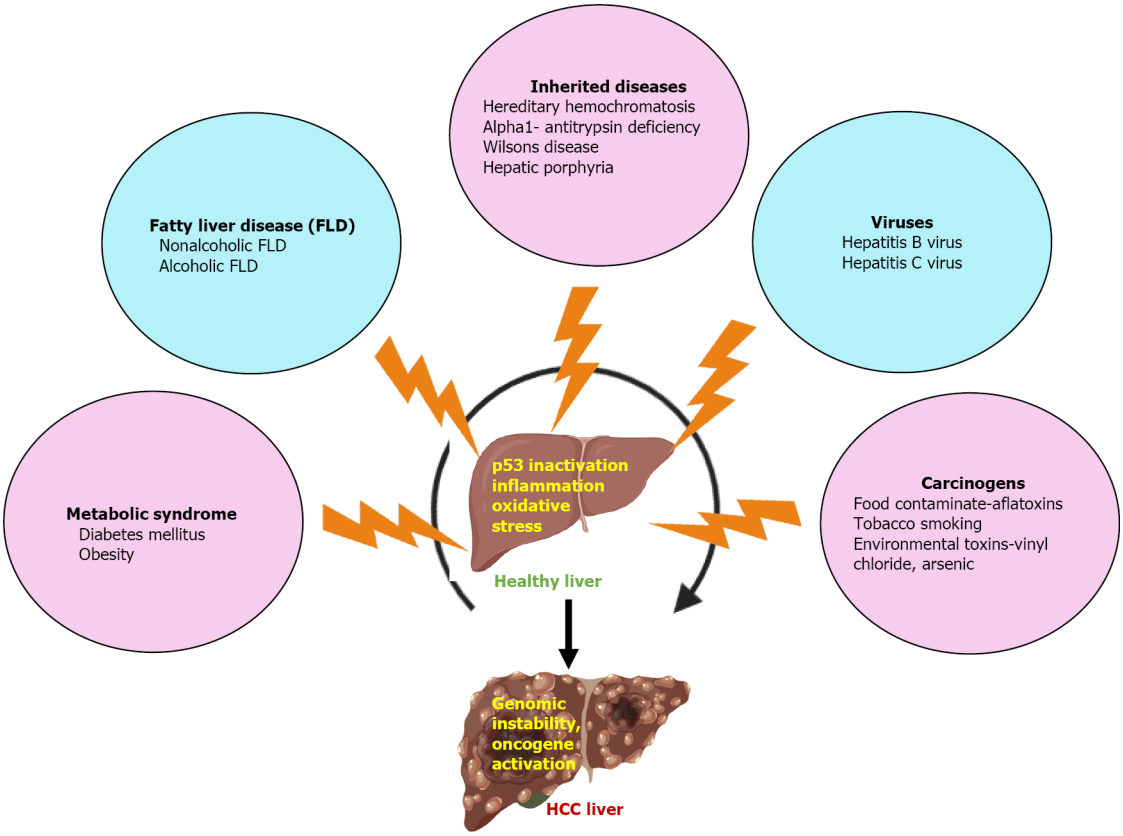
Hepatocellular carcinoma (HCC) is the world's fifth most frequent malignancy and the third main cause of cancer death[1]. Men are slightly more likely than women to get HCC (3:1 male-to-female ratio). Globally, primary liver cancer caused an estimated 906000 new cases and 830000 fatalities in 2020; the majority of these cases (75%-85%) were HCC[2]. Underlying liver conditions including cirrhosis or chronic hepatitis frequently led to HCC. Hepatitis C virus (HCV), hepatitis B virus (HBV), fatty liver disease like non-alcoholic steatohepatitis, and alcohol consumption are the most prominent risk factors (Figure 1)[3]. The 5-year age-standardized rate of relative survival for HCC is only 18.1%, even though surgical intervention is currently the best therapy for HCC. Tumor reappearance is common after tumor removal[4]. In adult males, HCC is the second most common cancer-related killer after lung cancer because of the challenge of earlier detection, which results in the vast bulk of HCC patients receiving a higher stage diagnosis at their first visit and being ineligible for treatments like radiofrequency ablation (RFA) or hepatectomy[5]. Sorafenib and lenvatinib, the two clinically authorized targeted therapy medicines, could barely extend survival time by 2 to 3 mo[6,7]. As a result, novel HCC therapy techniques are urgently required.

Immunotherapy is successful and safe in the treatment of solid tumors, resulting in long-term survival and manageable toxicity[8,9]. The liver is a remarkable immunologically tolerant organ that is exceptional in its capacity to accept liver transplants and diminish responsiveness to antigens in bacterial and food products through the portal vein[10]. This tolerogenic trait of the liver and the immunosuppressive tumor microenvironment of HCC may jointly inhibit the formation of anti-tumor immunity against HCC. Cancer immunotherapy may be a compelling therapeutic approach for HCC, which has a metachronous, multicentric incidence due to its capacity to trigger systemic and long-lasting anti-tumor actions. For numerous forms of malignancies, along with HCC, the FDA has so far approved seven immune checkpoint inhibitors (ICIs) that target the programmed cell death protein-1 (PD-1), proteins cytotoxic T lymphocyte antigen 4 (CTLA-4), or its ligand programmed cell death-ligand 1 (PD-L1)[11,12]. Other immunotherapeutic approaches, like immune cells modified with chimeric antigen receptors, adoptive cell treatment, tailored cytokines, and cancer vaccines, are almost ready and provide HCC patients new hope[13-15]. In this review, we first provide an overview of the current state of immunotherapy for HCC (Figure 2), after which we analyze the challenges, opportunities, and potential directions of this field of study.

ANTIBODY-BASED THERAPY

Monotherapy with ICIs

Effector immune cells express inhibitory immunoreceptors called immunological checkpoints that keep the immune system from overreacting. The T cell immunoreceptor with Ig and ITIM domains (TIGIT), lymphocyte-activation gene 3 (LAG3), B and T lymphocyte attenuator, and T cell immunoglobulin and mucin domain containing-3 (TIM3) are only a few of the inhibitory receptors in this group[16]. This



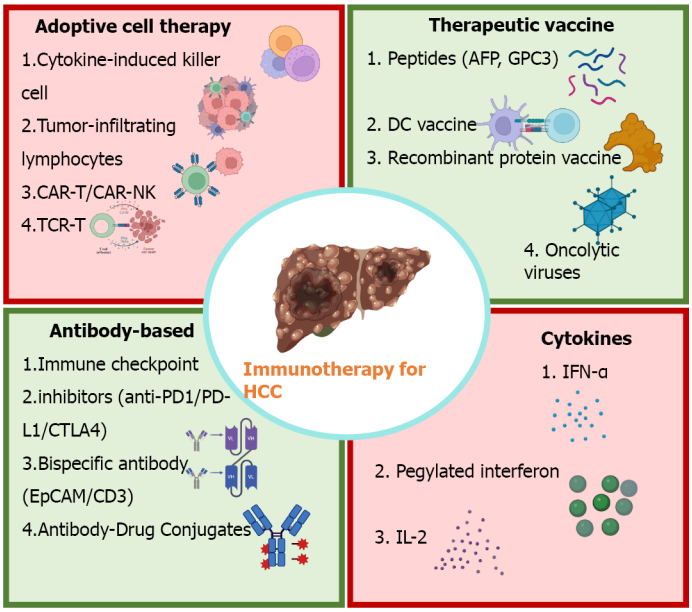
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Figure 1 Etiology of hepatocellular carcinoma. FLD: Fatty liver disease; HCC: Hepatocellular carcinoma.

physiological process is employed by HCC and other solid tumors to thwart anti-tumor immune reactions[17]. Monoclonal antibodies known as ICIs have the potential to prevent immunological checkpoint proteins from interacting with their ligands. This could improve the immune response against tumors by preventing T cell inactivation and reactivating immune attack and immune recognition. At the moment, ICIs' primary targets are CTLA-4, PD-1, and PD-L1[13]. Most immune cells, myeloid-derived suppressor cells, primarily activated T cells, regulatory T cells (Tregs), natural killer (NK) cells, dendritic cells (DCs), and monocytes express PD-1 and are members of the CD28 family. When PD-1 binds to its ligands, *i.e.* PD-L1 and PD-L2, that are stimulated in a variety of cancers, including HCC, it can send T cells inhibitory signals and cause tumor cells to bypass the immune system[18].

Nivolumab, a PD1 inhibitor, was given expedited approval in the United States in 2017 for this second-line treatment of patients with severe HCC following sorafenib therapy. ICIs have been used to treat HCC in several exploratory trials that have been completed so far. PD-1 and PD-L1 inhibitors pembrolizumab and atezolizumab, which target these receptors, respectively, have been steadily proposed as a clinical therapy strategy for HCC and added to the treatment recommendations of numerous nations. Nivolumab and pembrolizumab cause objective remissions of 15%-20%, which includes complete remissions of 1%-5%, and these remissions are long-lasting and linked with improved survival. Nivolumab had a median response duration of 17 mo in the dose-escalation cohort in 48 patients of the CheckMate 040 investigation, and responders had a survival rate of about 2 years of more than 80%[19]. Statistically longer survival was seen in the 413 patients who took part in phase III clinical trial KEYNOTE-240, which compared pembrolizumab after sorafenib medication to placebo. Pembrolizumab was found to have long-term advantages for some people, as seen by the overall survival curves and progression-free survival. Around 20% of patients who got pembrolizumab stayed in progression-free status for longer than a year, as opposed to merely 7% of the control group[20]. In the phase III CheckMate 459 trial, 743 patients who had never received systemic medications were compared between nivolumab and sorafenib. Patients who received nivolumab had a longer median survival time than those who received sorafenib[21]. The CheckMate 459 study's lengthier follow-up period confirmed nivolumab's better ability to sorafenib to raise the long-term rate of survival[22].

Recent studies presented at the ESMO 2021 Annual Meeting revealed that tislelizumab, a humanized monoclonal antibody (mAb) with substantial adhesion for PD-1, evidenced durable response and was well tolerated in individuals who had previously systemically treated unresectable HCC. In a global, randomized phase 3 trial, the first-line therapies for adult patients having unresectable HCC are tislel-



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Figure 2 Current immunotherapies for hepatocellular carcinoma. AFP: Alpha-fetoprotein; CAR-T: Chimeric antigen receptors engineered T cell; CTLA4: Cytotoxic T lymphocyte antigen 4; IFN: Interferon-alpha; IL-2: Interleukin-2; NK: Natural killer; PD-1: Programmed cell death-1; PD-L1: Programmed cell death-ligand 1.

izumab and sorafenib (NCT03412773)[23]. The majority of activated T cells and DCs express CTLA-4, a member of the CD28 family. After binding to B7 molecules, it participates in the downregulation of the immune response[24]. The FDA approved ipilimumab, the first ICI, in 2011 for the therapy of patients with severe skin cancer. Tremelimumab is another CTLA-4 inhibitor[25].

Tremelimumab, an IgG2 mAb, has different antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity activities than Ipilimumab, an IgG1 mAb[26]. Tremelimumab demonstrated a potent anti-HCC impact in a clinical trial conducted in 2013, with a 17.6% of partial response (PR) rate and 76.4% of disease control rate[27]. With the in-depth analysis of CTLA-4 inhibitors' mechanism, some researchers hypothesize that it involves the targeted eradication of Tregs from tumors rather than the immune checkpoint[28]. In human HCC, the presence of TIM3 on tumor-associated macrophages and tumor-infiltrating lymphocytes (TILs) impairs T cells' ability to operate as effector cells, while increasing the activation of Tregs' suppressor genes[29]. Less differentiated HCC is linked to the highly expressed TIM3[30]. LAG3 appearance is much greater on tumor-specific $CD4^+$ and $CD8^+$ TILs in patients with HCC compared to other immune compartments. Hepatocytes produce fibrinogen-like protein 1, another useful soluble ligand for LAG3[31]. The most recent studies published demonstrate that Siglec-15 inhibits CD44's lysosomal degradation, which encourages the migration of liver cancer cells[31,32]. T-cell immunoreceptor with immunoglobulin and ITIM domains also plays a role in TIGIT[33]. The TIGIT/CD155 pathway increases interleukin (IL)-10 synthesis and decreases IL-12 by DCs to prevent T cell activation[34]. The exploration of Siglec-15, TIM3, TIGIT, and LAG3 inhibitors in HCC in conjunction with PD1 and PDL1 inhibition is supported by the preclinical evidence presented here. According to the findings of current clinical studies, patients medicated with ICIs alone, have a lower rate of response; as a result, future therapies will combine ICIs and other therapies.

The results of the worldwide, randomized phase 3 IMbrave150 trial in 2020 showed that atezolizumab plus bevacizumab an anti-angiogenic drug considerably lowered the probability of mortality in patients with severe unresectable HCC and enhanced the rate of patient survival[35]. Lenvatinib, a tyrosine kinase inhibitor (TKI), was combined with pembrolizumab to provide an overall response rate of 46%. Patients with unresectable HCC who were enrolled in the study saw complete responses (CRs) and PR in proportions of 11% and 35%, respectively[36]. Similar to this, newer preclinical and clinical investigations have demonstrated that the administration of ICIs in combination with radiation, RFA, and transcatheter arterial chemoembolization (TACE) can enhance the effectiveness of anti-tumor immunotherapy[37,38]. In a phase, Ib/II clinical trial, camrelizumab and the chemotherapy FOLFOX4 are being used to treat advanced HCC[39].

The HIMALAYA phase 3 trial, which evaluated a single, high dose of tremelimumab, an anti-CTLA-4 antibody, added to durvalumab, an anti-PD-L1 antibody, or durvalumab alone in comparison to sorafenib as first-line treatment in 1171 patients with advanced HCC, has provided additional evidence that single-agent PD1/PD-L1 inhibition has antitumor activity (NCT03298451). Durvalumab monotherapy achieved the goal of overall survival without being inferior to sorafenib while being less toxic[40]. Regarding anti-CTLA-4 monotherapy, the research 22 revealed that tremelimumab

monotherapy in patients previously treated with sorafenib after unacceptable toxicity or rejection of sorafenib had a manageable safety profile. However, the benefit-risk ratio was often better when tremelimumab and durvalumab were used together[41].

Figure 3 depicts several ICIs in HCC. Table 1 presents a summary of the clinical trials including ICIs treatment for HCC conducted during the previous 3 years.

Dual therapies with ICIs

Combining PD-1 and CTLA-4 inhibitors: The concept of combining inhibitors of several immune checkpoints, including as PD-1 and CTLA-4, is now being investigated in advanced HCC. Dual treatments combining PD-1 and CTLA-4 inhibitors have been studied in other cancer types, such as melanoma. The CheckMate 040 trial, which evaluated nivolumab and ipilimumab, an anti-CTLA-4 antibody, in 148 patients with advanced HCC who had progressed on sorafenib, provided the first clinical data[42]. Nivolumab and ipilimumab were administered to patients in three separate arms (Arm A, Arm B, and Arm C) at various doses and frequencies. The trial reported a 49% disease control rate, a 31% overall response rate, and a 17-mo median duration of response. The longest overall survival was 23 mo for patients in arm A. The patient side-effect profile was deemed acceptable. To treat side effects, more than 50% of patients required systemic steroids. Overall, the trial's findings were positive, and the FDA eventually approved the use of nivolumab and ipilimumab together. Additionally, the combination is presently being evaluated in the CheckMate 9DW phase 3 study as a first-line treatment for advanced HCC in comparison to sorafenib or lenvatinib (NCT04039607). Similar to this, durvalumab and tremelimumab together exhibited strong action in unresectable HCC, with a response rate overall of 17.5%[43].

ICIs with anti-vascular endothelial growth factor antibodies: The combination of a PD1/PD-L1 inhibitor with a vascular endothelial growth factor (VEGF) inhibitor has been established as a novel paradigm for the treatment of advanced HCC in light of the encouraging results of the IMbrave150 phase 3 trial[20]. Atezolizumab plus bevacizumab had initially shown good safety and promising anticancer activity in patients with untreated advanced HCC in a phase 1b study[44]. The next phase of the IMbrave150 trial compared sorafenib and atezolizumab in patients with advanced HCC who had never received systemic therapy and were randomly assigned (2:1) to either study arm[20]. Compared to sorafenib, the combination of atezolizumab plus bevacizumab therapy lowered the death risk by 42%. The IMbrave150 trial, which is significant, excluded patients with problems related to portal hypertension, such as severe ascites and esophageal or gastric varices at a high risk of bleeding. This atezolizumab and bevacizumab combination, which is the current standard therapy that has replaced the TKIs sorafenib and lenvatinib, were approved as a first-line treatment for unresectable HCC in the United States and Europe as a result of the IMbrave150 trial. Inhibiting PD-L1, which activates the immune system (especially T-effector cells), and inhibiting VEGF, which lessens VEGF-mediated immunosuppression and encourages T-cell infiltration in the tumor microenvironment, are probable synergistic anticancer agents that account for its effectiveness[45].

Dual therapies combining immune checkpoint with multi-kinase inhibitors: An alternative to antibody-mediated VEGF suppression may be achieved by combining ICIs with TKIs instead of anti-VEGF antibodies. The multicohort COSMIC-021 phase 1b trial is evaluating the combination of cabozantinib and atezolizumab in advanced solid tumors, including HCC (NCT03170960). Additionally, it is being evaluated in the COSMIC-312 phase 3 trial (NCT03755791) as a first-line treatment for patients with advanced HCC in comparison to sorafenib[46]. As a secondary outcome metric, cabozantinib/monotherapy is also contrasted with sorafenib in this study. A predetermined interim analysis for overall survival, however, failed to achieve statistical significance. Early 2022 is when its final analysis should produce results[47]. In 104 patients with unresectable HCC who had not previously undergone systemic treatment, the combination of lenvatinib and pembrolizumab showed reasonable results in a phase 1b trial[36]. This combination is currently being compared to lenvatinib monotherapy in the LEAP002 phase 3 trial (NCT03713593)[48]. Finally, the combination of apatinib and camrelizumab, an anti-PD-1 antibody is under clinical development. An overall response rate of 50% was found in a phase 1 study of individuals with advanced HCC[49]. Additionally, a phase 3 trial is currently comparing the combination to sorafenib in the first-line setting for patients with advanced HCC (NCT03764293).

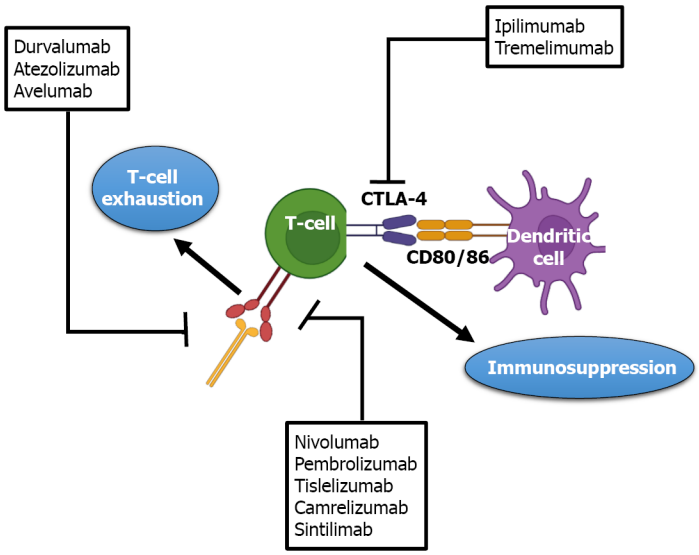
Bispecific antibody therapy

Bispecific antibody (BsAbs), in contrast to monoclonal antibodies, are made primarily using recombinant DNA technology and may concurrently and precisely bind two antigens or epitopes[50]. BsAb can target immunological checkpoints and tumor-associated antigens (TAAs) to alter immunosuppression in the tumor environment. It can also directly increase the action of immune cells against tumors. As a result, they outperform monoclonal antibodies in aspects of synergistic effects and can mediate a wide range of particular biological effects. Most of the time, BsAbs bridge the gap between immune cells and tumor cells to attract and activate them to attack tumor cells[51]. An EpCAM/CD3 bispecific antibody called Solitomab (AMG110, MT110) is humanized. Bispecific T-cell Engager (BITE),

Table 1 Immunotherapy with immune checkpoint inhibitor in hepatocellular carcinoma (clinical trial with reported results)

Treatment	Patients, *n*	ORR%	OS in mo	Ref.
Atezolizumab	59	17 (5)	NA	[44]
Nivolumab	371	15(4)	16.4	[21]
Camrelizumab	217	15 (0)	13.8	[165]
Pembrolizumab	278	18 (2)	13.9	[36]
Durvalumab	104	11 (0)	13.6	[46]
Tremelimumab	69	7 (0)	15.1	[46]
Durvalumab and tremelimumab	159	9.5-24.0 (1-2)	11.3-18.7	[46]
Pembrolizumab and levantinib	100	36 (1)	22	[20]
Nivolumab and ipilimumab	148	31-32 (0-8)	12.5-22.8	[42]
Atezolizumab and bevacizumab	336	27 (6)	NE	[20]
Nivolumab and cabozantinib	36	14 (3)	21.5	[42]
Nivolumab, ipilimumab and cabozantinib	35	31 (6)	NE	[42]

NA: Not available; NE: Not evaluable; ORR: Overall response rate; OS: Overall survival.



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Figure 3 Immune checkpoint inhibitors in hepatocellular carcinoma. CTLA4: Cytotoxic T lymphocyte antigen 4; PD-1: Programmed cell death-1; PD-L1: Programmed cell death-ligand 1.

which can lyse HCC cell lines almost completely *in vitro* when it binds to gd T cells, is created by joining the anti-EpCAM single-chain variable fragment (scFv) to the anti-CD3 scFv *via* a Gly4Ser linker[52]. To remove glypican-3 (GPC3) + HCC cells, a different BsAb called GPC3/CD3 BITE is expected to entice CTL. In one work, an IgG-shaped TriFab, that can be employed to activate two antigens sequentially or for targeted delivery of tiny and large payloads, was created by joining two anti-GPC3 Fab fragments to one asymmetric third Fab-sized binding module using flexible linker peptides[53].

ADOPTIVE CELL TRANSFER

Adoptive cell transfer (ACT), an immunotherapy that harnesses the patient's immune cells or those of a healthier donor to combat cancer, has currently emerged as a crucial component of cancer treatment [54]. ACT could be stimulated and replicated *in vivo* and has a long-lasting anti-tumor impact, in contrast to antibodies or other targeted medications. As a result, the ACT is also known as a "living"

therapeutic strategy[55]. Since the majority of ACT's effector cells come from the patient, it is regarded as a highly personalized cancer therapy. ACT is more targeted than chemotherapy because enlarged or genetically altered effector cells can identify and target tumor antigens[56]. Adoptive cell transfer, as opposed to checkpoint inhibition therapy, which passively administers autologous lymphocytes after ex vivo cultivation, aims to improve the outcomes for patients with HCC[57]. Table 2 lists the ACT clinical studies for the HCC treatment that were published at clinicaltrials.gov in the previous 3 years.

Cytokine-induced killer cell: IL-2, interferon-alpha (IFN)-α, and anti-CD3 monoclonal antibodies are used to create cytokine-induced killer cell (CIK) cells, a heterogeneous population of immune cells, from human peripheral blood mononuclear cells (PBMC) in a laboratory setting[58]. NK cells, NKT cells, and CTLs make up the majority of CIK cells (CTLs). Through adhesion molecules, CIK can lyse tumor cells and recognize them without the assistance of the major histocompatibility complex (MHC). Shi *et al*[59] showed that autologous CIK cells can effectively enhance the immunological condition of HCC patients by using them to treat primary HCC in a phase I clinical study and finding that the signs and features of HCC patients were diminished without notable adverse effects. Clinical studies have also demonstrated that CIK cell treatment could be utilized to cure individuals having primary HCC that is inoperable as well as HCC patients who have had their tumors removed. Takayama *et al*[60] published the results of a 150-patient clinical trial of CIK therapy for postoperative HCC. They discovered no significant treatment adverse effects and an 18% lesser recurrence rate in the treatment group than in the control group, indicating that CIK cells treatment can lower reappearance rates and lengthen recurrence-free survival in patients with postoperative HCC[61]. Additionally, conventional therapies and CIK cell therapy have frequently been combined by researchers. As opposed to TACE alone, TACE combined with CIK cells may increase progression-free survival in HCC patients. T and NKT cells dramatically increased with the addition of CIK cells to local radio frequency hyperthermia, whereas alpha-fetoprotein (AFP) levels decreased, according to research by Wang *et al*[61]. Although 17% of patients reported experiencing pyrexia, chills, myalgia, and fatigue, these symptoms were not severe enough to require therapy to be stopped[62]. These results suggest that the combination of CIK cells having TACE or RF hyperthermia is a harmless and efficient therapy for HCC patients.

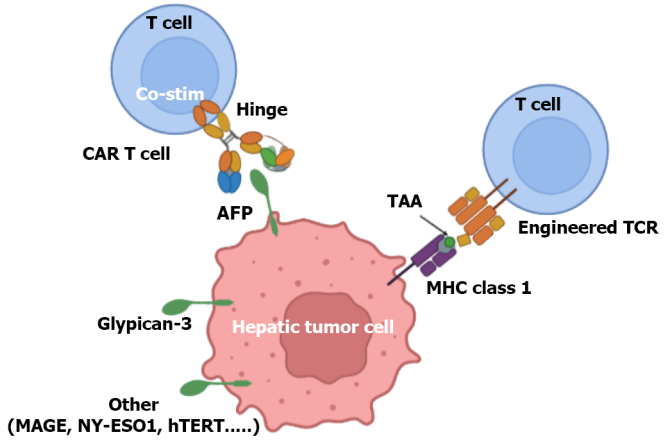
Tumor-infiltrating lymphocytes: TIL is a part of the host immune response against tumors, which also consists of Tregs, T cells, NK cells, and B cells[63]. TIL is 50-100 times more effective as compared to lymphokine-activated killer cells at curing mice with severe metastatic cancer, according to experiments [64]. A phase I clinical trial in individuals with primary HCC established the viability of TIL treatment [65]. Because TILs are derived from surgical tumor tissues and can detect several antigens, they have a stronger tumor-inhibitory effect than medications that target certain antigens or mutations. TILs in HCC are uncommon, however, prior research has shown that despite this, they can significantly affect tumor recurrence and patient prognosis[66]. Clinical research with 150 HCC patients demonstrated that adoptive TIL treatment improved recurrence-free survival following liver resection[60]. Patients who received surgical resection of their HCC and had considerable lymphocyte infiltration had a lower recurrence rate and a greater survival rate than those who did not have significant lymphocyte infiltration[67]. However, isolating TILs from HCC tumor tissues and expanding them *in vitro* is difficult. Additionally, only a small number of HCC patients could survive lymphocyte deletion, which is necessary before TIL infusion[68].

Chimeric antigen receptor T Cell: ACT research is now focused on Chimeric antigen receptor T Cell (CAR-T) treatment, novel immunotherapy for cancer wherein T cells are genetically altered to recognize particular TAA[69]. Hematological cancers can be successfully treated with CAR-T cell therapy. The FDA has approved CAR-T cells that target CD19 and B-cell maturation antigen (BCMA) for the management of acute B-cell lymphocytic leukemia, several lymphomas, and multiple myeloma[70]. CAR-T therapy for liver malignancy is currently being developed because of the variety of solid tumors, the absence of specific targets, and the liability to the tumor microenvironment[71]. Conventional T cells have a T cell receptor (TCR) structure that is reliant on MHC antigen presentation. The CAR structure is independent of MHC antigen presentation, evades MHC molecule restriction, and resolves the issue of tumor immune escape caused by MHC downregulation[72] (Figure 4). To date, an increasing series of clinical trials have been carried out to show the effectiveness of CAR-T cell treatment in solid tumors. GPC3, a 580 amino acid long heparan sulphate proteoglycan, is overexpressed in HCC yet not at all in healthy tissues[73,74]. Gao *et al*[75] created CAR-T cells targeting GPC3 for the first time and showed that GPC3 CAR-T cells can successfully eradicate the proliferation of HCC cells both *in vitro* and *in vivo*. In a recent study, researchers found that by dividing the CAR design into two parts, HCC tumors may be removed with a lower level of proinflammatory cytokines (split GPC-3 CAR-T cells)[76]. A different study used patient-derived xenograft (PDX) HCC models and showed that GPC3 CAR-T cells reduced tumor development, however to differing degrees because of variations in PDL1 expression on cancer cells[77]. This demonstrates that combining CAR-T treatment and ICIs is a workable way to increase the efficiency of curing PD-L1- +ve HCC. The secreted glycoprotein known as AFP is extensively expressed in fetuses but only modestly so in adults. However, AFP is once again expressed when HCC manifests in adults[78]. Traditional CAR-T cells are only able to identify tumor surface antigens; they are unable to identify intracellular antigens. Liu *et al*[79] developed some distinctive CAR-T cells that could bind to

Table 2 Advantages and disadvantages of vaccination strategies used in hepatocellular carcinoma

Vaccine type	Advantages	Disadvantages
Peptides	Easy to prepare; known target Ag	Adjuvants are needed; restricted Ag repertoire; restriction to human leukocyte Ag
Dendritic cells	Adjuvants are not needed	Individualized production
Peptide pulsed	Known target Ag	Restriction to human leukocyte Ag; restricted Ag repertoire
Protein pulsed	No restriction to human leukocyte Ag; known target Ag	Protein synthesis is more interesting; restricted Ag repertoire
Tumor lysate pulsed	Not human leukocyte Ag restricted full Ag repertoire; available	Tumor samples not always available; the predominance of self-Ags that may eclipse tumor Ags
Cell line pulsed	Not human leukocyte Ag restricted; unlimited Ag source	Responses against cell line-specific Ag

Ag: Antigen.



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Figure 4 Schematic representation of T cell receptor engineered T cells and chimeric antigen receptors (CAR) engineered T cells in hepatocellular carcinoma. AFP: Alpha-fetoprotein; CAR-T: Chimeric antigen receptors engineered T cell; MAGE: Melanoma-associated antigen; MHC: Major histocompatibility complex; TAA: Tumor-associated antigens; TCR: T cell receptor.

the AFP158-166 peptide-MHC complex and then damage HLA-A*02:01+/AFP+ tumor cells because all intracellular antigens are delivered by MHC class I molecules. A phase I clinical trial (NCT03349255) assessing the security and performance of CAR-T cells in patients with AFP-expressing HCC was successfully finished in the interim. As a result, using CAR-T cells to target intracellular antigens is an effective way to treat HCC. The c-Met tyrosine kinase receptor could promote hepatocyte survival, regeneration, and proliferation[80].

HCC development and progression can be aided by c-Met overexpression. As a result, c-Met is being evaluated as a possible target for the therapy of HCC. Jiang *et al*[81] developed CAR-T cells that simultaneously targeted c-Met and PD-L1, and they discovered that these cells significantly cytotoxic attacked c-Met+ PD-L1+ HCC cells. NKG2DL ligands (NK group 2 member D ligands) are present in a variety of primary malignancies, along with HCC, yet not found in healthy tissues[82]. Therefore, NKG2DL might be a good candidate for HCC immunotherapy. Innovative NKG2D-CAR-T cells that specifically target NKG2DL expressed on HCC cells were developed by Sun *et al*[83]. They also discovered that NKG2D-CART cells only targeted ruptured HCC cells with elevated NKG2DL expression and had no effect on the NKG2DL negative cell line. The xenograft model further demonstrated that NKG2D-CAR-T cells may prominently limit *in vivo* tumor growth. Type I transmembrane glycoprotein CD147 was discovered in high levels in HCC and various solid tumors [84]. When Tet-On inducible CD147-CART cells were administered Dox, Zhang *et al*[85] found that Tet-On inducible CD147-CART cells can successfully rupture numerous HCC cell lines *in vitro* and reduce the development of cancer cells in the HCC xenograft model. The safety of hepatic artery infusions (HAI) of CD147-CART cells for severe HCC was recently evaluated in a phase I trial (NCT03993743). Mucin 1[86], EpCAM[87], and CD133 are further potential targets antigens for HCC CAR-T treatment [88,89]. The majority of the above-stated targets, though, are TAAs, which are expressed at low levels in

both cancerous and healthy cells, leading to both on- and off-target toxicities in normal tissue. The most significant objective for future researchers will be to identify new targeted antigens and improve the effectiveness and safety of CAR-T treatment for HCC.

Chimeric antigen receptor NK cells: The distribution of NK cells in the liver is substantially greater as compared to the peripheral blood and spleen. As a result, NK cells are thought to be important in the prevention of HCC and are assumed to be a promising source for cell therapy in the treatment of HCC [90]. The technique utilized to produce CAR-T cells could likewise be employed to generate CAR-NK cells from NK cells. Furthermore, because CAR-NK cells have a shorter lifespan as compared to CAR-T cells, they can lower the danger of autoimmune response and tumor transformation[91]. CAR-NK cells could also be generated from various resources, such as the peripheral blood mononuclear cells (PBMC), NK92 cell line, umbilical cord blood (UCB), and induced pluripotent stem cells (IPSC). Since CAR-NK cells could be delivered "off-the-shelf", abolishing the requirement for customized and patient-specific products, so is the case with CAR-T therapies currently, the danger of syngeneic xenograft responses and graft-versus-host (GVHD) sickness is reduced[92]. The therapeutic potential of GPC3-specific CAR-NK cells for HCC was investigated by Yu *et al*[93] in 2018. *In vitro* co-culture experiments with $GPC3^+$ HCC cells revealed considerable cytotoxicity and cytokine generation by GPC3-specific CAR-NK cells. Additionally, cytotoxicity was unaffected by soluble GPC3 and TGF-b, and hypoxic (1%) conditions did not significantly alter anti-tumor efficacy. In a different study, Tseng *et al*[94] developed CD147-specific CAR-T and CAR-NK cells to treat HCC using CD147 as the target antigen. The results indicated that numerous malignant HCC cell lines *in vitro*, as well as HCC tumors in xenograft and PDX animal models, could be successfully eliminated by CD147-specific CAR-NK cells. In a human CD147 transgenic mouse model, GPC3-synNotch-inducible CD147-specific CAR-NK cells preferentially destroy $GPC3^+CD147^+$ however not $GPC3^-CD147^+$ HCC cells and don't induce substantial on-target/off-tumor damage. The lack of effective gene transfer techniques in primary NK cells is one of the fundamental obstacles to CAR-NK immunotherapy. Numerous current investigations have shown that retroviral vectors may successfully transduce larger NK cells with efficiency ranging around 27% to 52% after just one round of transduction[95]. The insertional mutations linked to retroviral transduction and the detrimental actions on primary NK cell survival, however, are the most significant drawbacks of this strategy in a clinical setting.

TCR-engineered T Cell: TCR-Engineered T Cell (TCR-T) cells are created by altering T cells with the exogenous TCRs gene to precisely identify tumor antigen peptides-MHC complexes[96]. All tumor-derived proteins could indeed be broken down *via* proteasomes and displayed by MHC, allowing TCR-T cells to target both tumor surface and intracellular antigens. TCR-T therapy should therefore be more widely applicable compared to CAR-T therapy. HCV infection affects 130-150 million people worldwide and could result in conditions like HCC[97]. Spear *et al*[98] engineered T cells with a high affinity, HLA-A2-restricted, HCV NS3:1406-1415-reactive TCR to create HCV-specific TCR-T cells. The findings demonstrated that HCV-specific TCR-T cells may cause a relapse of established HCV+ HCC *in vivo*, indicating that HCV-specific TCR-T therapy might be an efficient technique for curing HCV-associated HCC. A lesser proportion of HCC tissues produced from HBV infection detain HBV gene expression, which may become TCR-T targets. In 2011, Gehring *et al*[99] identified TCR-T cells specific for the HBV surface antigen in PBMC of patients with chronic HBV and HBV-related HCC. These HBV-specific TCR-T cells may recognize HCC tumor cells and have a variety of uses.

Additionally, a phase I clinical experiment was carried out to assess the safety and efficiency of HBV-specific TCR-T in stopping HCC reappearance following transplantation of the liver[100] (NCT02686372). As previously stated, AFP is another TAA linked to HCC. Docta *et al*[101] recently mentioned the discovery of a human HLAA2/AFP158-specific TCR, and AFP-specific TCR-engineered autologous T cells from HCC patients are now being used in a clinical investigation (NCT03132792). In 2018, we found a large number of HLA-A2/AFP158-specific TCRs from HLA-A2 transgenic mice using an immunization technique that comprised recombinant lentiviral priming and peptide boosting[102]. These TCRs were expressed on human T cells, and both *in vitro* and *in vivo* tests revealed strong anti-tumor efficacy. Additionally, the outcomes of comprehensive X-scans showed that there is little to no cross-reactivity between these TCR T cells and human cells. A clinical trial including these TCRTs for the treatment of HCC patients has begun (NCT03971747). GPC3[103], New York esophageal squamous cell cancer 1 (NY-ESO-1)[104], and human telomerase reverse transcriptase (hTERT) are further options for HCC TCR-T treatment[105]. However, because TCRs are promiscuous, TCR-T cells might cross-react with healthy tissue MHC-peptide complexes, resulting in off-target damage. TCRs produced in mice and humans can both cause off-target harm.

Despite being produced in humans, the melanoma-associated antigen (MAGE)-A3/HLA-A1TCR induced severe cardiac damage *via* targeting the cardiac muscle protein titin[106]. However, while NY-ESO-1 TCRT has demonstrated clinical anti-tumor efficacy, the majority of similar TCRTs haven't been confirmed to be useful for patients. Various aspects could be addressed to increase the anti-tumor efficacy of TCR-T therapy, such as extending TCR-T *in vivo* survival, enhancing tumor infiltration, and preventing T cell depletion.

CYTOKINES RELATED TO ADVANCED HCC

Key elements of the immune system, cytokines are crucial in the immunological response to cancer. Recently, there has been a lot of interest in using cytokines to treat cancer as the immune system is competent in identifying and eliminating cancer cells[107]. In 1986, the United States FDA approved the use of IFN-α as the first cytokine for the treatment of hairy cell leukemia (HCL)[108]. In 1992, the use of high-dose IL-2 as a therapy for metastatic renal cell carcinoma (mRCC) and metastatic melanoma was approved (MM). Besides its original approval, IFN-α has been used to treat a variety of cancers, including mRCC, melanoma, follicular lymphoma when combined with bevacizumab, and Kaposi's sarcoma linked to the AIDS virus. Numerous factors can be used to categorize advanced diseases in HCC. The major liver disease societies approve the BCLC staging, which has been well verified[109]. In the BCLC staging system, level C is the advanced stage, and stage D is the final stage[110]. Multiple cytokines and stimulatory substances have been linked to the likelihood of progressive disease in HCC patients.

Interleukin-10: IL-10 is a highly effective anti-inflammatory cytokine[111]. The majority of activated immune cells, along with macrophages and monocytes, secrete IL-10, which reduces the secretion of inflammatory mediators, prevents the formation of antigens, and controls several other immunological properties[112]. While its significance in viral infections is widely known, its function in HCC is less clear. Interpreting IL-10 data for HCC is complicated by the finding that HCC patients have greater levels of IL-10 than cirrhotic patients and healthy controls, although not in viral hepatitis patients[113]. In one investigation, it was discovered that patients with IL-10 levels of more than 12 pg/mL had inferior post-operative results. It has also been investigated how IL-10 affects HCC which is incurable. IL-10 levels in serum were revealed to be a poor prognostic predictor in a prospective analysis of 74 patients with unresectable HCC, with a considerably poorer median survival (3 mo *vs* 12 mo; $P = 0.02$) [114]. Patients with elevated IL-10 levels in serum performed markedly worse than patients with decreased IL-10 levels in a larger trial of 222 patients with unresectable HCC (mainly related to HBV) [hazard ratio (HR) 2.2][114]. Elevated IL-10 levels dramatically decreased overall survival in patients with advanced disease (BCLC stage C) from 10.2 mo to 3.5 mo, compared to those with low IL-10 levels, who exhibited average mortality of 10.2 mo[115].

Interleukin-37β: The wideset of the 5 distinct isoforms of IL-37β (named IL-37a-e) is IL-37β[116]. This cytokine, which is generated by epithelial cells, macrophages, and monocytes inhibits the production of pro-inflammatory cytokines as well as EMT by activating the STAT3/IL-6 pathway[117]. Additionally, recombinant IL-37β studies *in vivo* on mice showed that the tumor volume was lowered compared to the untreated controls[118]. IL-37 β serum levels were found to be negatively correlated with the survival of advanced HCC in a study of HBV-related HCC patients. Higher levels of IL-37β in the subjects' bodies led to prolonged overall survival and disease-free survival[119]. Similarly, in a cohort with a high percentage of HBV-HCC, higher IL-37β expression in HCC tissues was linked with better DFS and survival[118]. These HCC findings, along with decreased IL-37β expression and production in metastatic tumors, suggest that IL-37β is implicated in signaling pathways that control metastasis and may have an impact on histopathologic prognosis[119].

CC Chemokine Ligand 20: CC Chemokine Ligand 20 (CCL20) (also known as macrophage inflammatory protein-3 alpha) links with CC chemokine receptor 6 (CCR6), causing immune cells to be chemoattracted to the zone of inflammation. CCL20 have been demonstrated to play several functions in rheumatoid arthritis, general inflammation, and various cancers[120]. *In vitro* and *in vivo* studies have revealed a function for the CCL20-CCR6 axis in HCC generation, development, and invasion[121]. Furthermore, in an investigation of 33 specimens from 22 patients, CCL20 overexpression was detected in tumors, indicating a function in hepatocarcinogenesis[122]. A role in tumor invasion, angiogenesis, and the development of hepatic malignancies has been suggested by several studies that have detected significant amounts of CCL20 and its receptor CCR6 in HCC and colorectal cancer liver metastases. The relationship between CCL20 expression and tumor grading, however, was only identified in one small study including 11 HCC patients (TNM stage 3 *vs* 2)[123]. Tumor-infiltrating regulatory T cells may be preferentially attracted to the tumor *via* the CCR6-CCL20 axis, according to an investigation of 293 HCC patients. This study found a favorable correlation between the number of regulatory T cells infiltrating the tumor and CCL20 expression in the tumor. Significantly, patients with HCC who had larger levels of tumor-infiltrating regulatory T cells had a worse prognosis[124].

IL-6: The predictive importance of pre-treatment blood IL-6 levels in the condition of advanced HCC was examined in research on 128 sorafenib-treated HCC patients divided into a finding and validating cohort. In both groups, a high level of serum IL-6 before therapy was a reliable indicator of poor overall survival. The time to progression and progression-free survival were comparable independent of pretreatment IL-6 levels, hence there was no association with sorafenib efficacy. Furthermore, pretreatment IL-6 levels were not associated with macrovascular invasion or extrahepatic dissemination [125]. Although promising, further research which is presently being undertaken, is required to establish the function of IL-6 in HCC therapeutic response. Interestingly, recent research in cellular

models has shown that blocking IL-6-related pathways reduces sorafenib resistance[126].

Angiopoietin-2: A major regulator of vascular development, angiopoietin-2 (ANG-2) is nearly entirely generated by epithelial cells and supports the actions of other endothelial-acting cytokines[127]. Superior pretreatment in the SHARP research, the first randomized placebo-controlled experiment to assess the effect of sorafenib in advanced HCC as well as the prognostic significance of multiple cytokines, ANG-2 levels were associated with shorter overall survival in the overall cohort as well as in the sorafenib arm. However, no link was discovered between sorafenib-associated survival and therapy interaction analyses. Nonetheless, those with higher plasma ANG-2 levels at week 12 had a smaller total survival and time to progression than those who did not have an increase in plasma levels[128]. A year after, the Okayama Liver Group (Japan) then performed a longitudinal analysis and retrospective investigation of serum cytokines in 2 independent sorafenib-treated advanced HCC cohorts. Increased pretreatment ANG-2 levels, as in the SHARP research, were linked to a worse overall survival rate[129]. Furthermore, when the researchers assessed ANG-2 in a prospective cohort, the difference was not statistically significant, most likely because there were fewer patients, even though patients with progressive disease had higher ANG-2 levels at the beginning of treatment than those having the non-progressive disease. ANG-2 levels, on the other hand, increased only in patients with worsening disease over time[129].

Hepatocyte growth factor: *In vitro* and animal models have revealed that hepatocyte growth factor (HGF) could either promote or prevent the development of HCC[130]. In both the sorafenib arm and the overall cohort of the SHARP trial, increased pretreatment plasma HGF levels were a significant independent predictor of worse overall survival. Surprisingly, sorafenib was linked with a greater benefit in terms of total survival and time to progression in patients with reduced HGF amount at the start of therapy. Additionally, in the therapy arm, a reduction in median HGF plasma levels at 12 wk was not associated with overall survival but was associated with a lengthier duration of progression [128]. In a prospective cohort, the Okayama Liver Group discovered pretreatment serum HGF levels to be a potential independent marker of overall survival, albeit significance was lost following multivariate analysis. Additionally, only in the retrospective cohort, HGF pretreatment levels were higher in patients with the progressive disease compared to non-progressive disease[131].

VEGF: Multityrosine kinase inhibitors, such as Sorafenib, target VEGF signaling as a major cytokine causing angiogenesis. The SHARP study looked at VEGF as a predictive marker in addition to ANG-2 and HGF. Similar to ANG-2, higher pretreatment VEGF amounts were linked to reducing survival. Nevertheless, the Sorafenib arm did not convert its predictive value. Interesting, the Sorafenib group's average plasma VEGF levels were significantly greater than those of the placebo group[128]. Furthermore, VEGF levels were shown to be higher in individuals than those later experienced disease advancement compared to non-disease progression in a retrospective study on HCC patients administered with sorafenib by the Okayama Liver group. Furthermore, higher baseline VEGF levels were linked to shorter overall survival and progression-free survival, which is consistent with the results of the SHARP trial. Nevertheless, the multivariate analysis failed to identify VEGF as a predictor of total survival[131]. Later, the same study team verified this finding in a prospective cohort of HCC patients receiving sorafenib therapy[129]. A decrease in plasma VEGF levels at 8 wk from baseline was shown by Tsuchiya *et al*[132] to be an independent predictive factor linked with 1-year survival following Sorafenib therapy in a small cohort of HCC patients.

THERAPEUTIC VACCINE

Tumor vaccines are substances that boost particular immune reactions to tumor antigens. For patients whose tumors don't have a lymphocytic infiltration that can be treated with ICI, immune-enhancing techniques may be helpful. One of the earliest immunotherapeutic methods applied in HCC among these methods is vaccination. In comparison to studies looking at adoptive cellular therapies and checkpoint inhibitors, clinical trials for such tumor vaccines in HCC are presently being conducted far less frequently. This is partly due to earlier disappointing trial results as well as the relative ineffectiveness of other tumor vaccines. This could be because it was formerly challenging to identify the proper tumor antigens, but that is now possible because of recent technology advancements permitting huge parallel DNA sequencing. Therefore, inducing an immune response remains a promising treatment strategy for HCC, either by itself or more likely in combination including an immune modulator. To date, several agents have been looked at in this area.

The high recurrence rate cannot be avoided by current treatments for advanced-stage HCC due to their insufficient efficacy. Vaccines have already been suggested as potential solutions to this problem, able to enhance clinical results when used in conjunction with existing recognized systemic treatments. Though they are harmless and have immunologic effects, the few trials that have been undertaken so far have only shown disappointing/poor clinical results/efficacy[133]. The majority of them are quite old,

and they are all phase I or II trials (Table 1). Peptide-based or DC-based vaccines are two categories of HCC vaccination methods now in use (Table 1). Additionally, this could be further divided into peptide-loaded and tumor lysate-pulsed DCs. Epitopes from the oncofetal antigens AFP[134,135], glypican 3 (GPC-3)[136], and the hTERT peptide GV1001 are the principal antigens used for peptide-based vaccinations in HCC[137]. Additionally, peptides from AFP and AFP coupled with MAGE-1 and GPC3 have been tested in clinical trials utilizing DCs loaded with peptides[138]. HepG2 (a hepatoma cell line) and autologous tumor lysates have both been employed in clinical trials[138,139]. All of these vaccination methods are risk-free, and even though clinical outcomes were subpar, the majority of them produced antigen-specific immune reactions without inducing toxic or autoimmune reactions. The various characteristics of HCC tumors and the vaccine's design, or even the combination of the two, may be to blame for this poor performance.

TAA-based vaccines are prone to tolerance mechanisms because they are not entirely tumor specific, which is reflected in the dearth of highly reactive clones against them[140]. As a result, responses are typically insufficient to stop tumor development. Additionally, as previously mentioned, the immunosuppressive HCC environment is not favorable for immune responses since it is a clear, prominent reflection of the intrinsic hepatic environment[141]. In addition, the restricted amount of TAAs expressed in HCC tumors makes it possible for immune evasion through Ag loss. Regarding their manufacturing, antigenic repertoire, and the variety of patients to be cured, the various vaccination modalities employed in HCC patients each have their benefits and drawbacks, which are listed in Table 2. Clinical trials for HCC have not yet fully utilized all vaccine strategies. Vaccination clinical trials in HCC with reported results are shown in Table 3. By changing the vaccination platform or adding new tumor antigens, vaccines may be improved. The first entails techniques like tumor cell fusion and *in vivo* DC-targeted immunization[140,141]. The assumption behind targeted vaccines is that by linking the antigen to antibodies, ligands, or viruses, non-target cell Ag delivery is prevented, potentially reducing any negative effects. In a recent preclinical model, we showed that this tactic increases the therapeutic effectiveness of ICI if it is paired with other treatments[142]. WT-1, ROBO1, FOXM1, and NY-ESO-1 are additional TAAs that may be utilized in upcoming clinical studies about a larger antigenic repertoire [143,144]. Finally, future HCC anticancer vaccination strategies should take into account neoAgs because of their tumor selectivity and possible increased immunogenicity[145].

DCs

DCs are expert antigen-presenting cells with a wide range of responsibilities, including the processing, presentation, and absorption of TAAs. Allogeneic DCs make up a significant portion of vaccines because they provide the antigen as well as the secondary co-stimulation required to effectively elicit a T cell activation. DCs from peripheral blood are isolated, grown ex vivo, and activated with cytokines like granulocyte-macrophage colony-stimulating factor (GM-CSF) to produce primed DCs for reinfusion. Another appealing, targeted strategy involves injecting such cells to cause the activation of effector cells, trigger a cascade of tumor rupture, and cause more TAA secretion[146]. To boost the effectiveness of the vaccine and optimize this TAA priming, a variety of approaches may be used. DCs might be cultured with tumor lysate or the fusion of DCs and tumor cells, or they may be transduced with DNA or RNA encoding recognized TAAs[141]. Ilixadencel (pro-inflammatory allogeneic DCs activated by GM-CSF and IL-4) was injected intratumorally in a newly published phase I trial, where 17 patients received it either alone or in conjunction with sorafenib. Tolerability evaluation was the main goal. There was just one grade 3 adverse event noted. Enhanced tumor-specific $CD8^+$T cells were seen in the peripheral blood of 73% of the 15 evaluable patients, indicating at least a successful immune-provoked response [147].

Peptide vaccines

An alternate choice for eliciting a powerful immune response is peptide vaccinations. However, despite immunological surrogates, like the generation of GPC-3 reactive cytotoxic T cells in one phase I trial, being successful, this has not led to clinical results[148]. GPC-3 and MRP3 have shown any efficacy in inducing a T cell response rate over 70%, while other TAAs such as SSX-2, NY-ESO-1, hTERT, and MAGE-A all induce considerably lower rates. This is despite a plethora of TAAs being found in HCC trials using AFP[149].

Oncolytic viruses

A more recent development in the arena of tumor vaccines is the use of oncolytic viruses. These therapeutically useful viruses are targeted to preferentially replicate in cancer cells. To date, they have been predominantly introduced by intra-tumoral injection. The modified poxvirus JX-594 remains the lead oncolytic virus of interest in clinical trials about HCC. As an immunotherapeutic agent, it piqued considerable interest when it conferred a dose-related survival benefit (median of 14.1 mo compared to 6.7 mo) in a phase II dose-finding trial of 30 patients[150,151]. The global, randomized, open-label, phase III study of Pexa-Vec (JX-594; an oncolytic vaccinia virus which selectively targets cancer cells) is currently recruiting patients with advanced HCC to two arms of vaccination with sorafenib *vs* sorafenib alone[152]. We excitedly await the results of this, particularly as a combination therapy.

Table 3 Vaccination clinical trials in hepatocellular carcinoma with reported results

Vaccine	Patient inclusion criteria	Patients, *n*	Immune response, %	Observations	Ref.
AFP HLA-A*02 restricted peptides + IFA	AFP + tumors from (stage IV patients)	6	66	Increased CTL response	[134]
DCs pulsed with autologous tumor lysate	Unresectable HCC	8	62	Immune response generation	[152]
DCs pulsed with autologous tumor lysate	Advanced HCC	31	0	Improved survival	[138]
DCs pulsed with AFP HLA-A*02 restricted peptides	Stage IV patients with surgery chemotherapy	10	60	No clinical responses	[135]
DCs pulsed with hepatoma cell-line (HEP-G2) lysate	No other therapeutic option	35	11.4	Evidence of antitumor efficacy	[139]
Gv1001 peptide + GM-CSF + cyclophosphamide	Advanced-stage HCC with no previous antitumor treatment	37	0	The immunological response is not detected	[137]
GPC3 HLA-A*24:02 and HLA-A*02-restricted peptides + IFA	Metastatic HCC or advanced HCC	33	91	Antitumor efficacy	[148]
DCs pulsed with fused recombinant proteins (AFP, MAGE-1 and GPC-3)	Surgical resection and locoregional therapy	12	92	Improved survival	[65]
GPC3 HLA-A*24:02 and HLA-A*02-restricted peptides + IFA	Vaccines as adjuvant therapy	41	85	Improved recurrence rate	[136]
AFP HLA-A*24:02 restricted peptides + IFA	Stage B/C tumors	15	33	Increased CTL response	[154]

Clinical trials with no published results have been excluded from the review. Incomplete Freund's Adjuvant, *immunologic response measurement was not appropriate. AFP: Alpha-fetoprotein; CTL: Cytotoxic T lymphocyte; DC: Dendritic cell; HCC: Hepatocellular carcinoma; GPC-3: Glypican-3; IFA: Incomplete Freund's Adjuvant; MAGE: Melanoma-associated antigen.

Immunotherapy toxicities and management

Immune-related adverse events: Immune checkpoint molecules serve an important function in immune homeostasis. Inhibitory immune checkpoint molecules, like PD-1 and CTL-4, are particularly important for balancing activation of T-cell and self-tolerance[153-155]. ICIs targeting PD-1 or CTL-4 may thereby produce a range of immune-related adverse events (IrAEs) by increasing self-immunity. IrAEs can affect every organ system and can range from a minor rash to life-threatening consequences. IrAEs caused by PD-1/PD-L1 inhibition are dose-independent[156]. Unlike anti-PD-1/PD-L1 agents, the risk of antiCTL-4-related adverse events is dose-dependent[157]. The gastrointestinal tract and skin were the most often affected organ systems by both anti-CTLA-4 and PD-1/PD-L1 inhibitors, whereas the endocrine systems and liver were less frequently affected[156,157]. In meta-analysis of phase 2/3 trials patients treated with ICIs, found that a rash was the most prevalent all-grade IrAE, with colitis and an increase in aspartate aminotransferase level being the most common high-grade IrAEs. Ipilimumab was related with a significantly greater incidence of rash and colitis when compared to anti-PD-1/PD-L1 drugs[158].

Management of IrAEs: Because IrAEs are linked with a wide range of aggravating factors in the context of HCC, hepatologists face significant hurdles in detecting and managing them. First, liver cirrhosis causes progressive immunological dysfunction, including immune deficiency as well as systemic inflammation[159]. Thus, in these patients, the liver-related immune homeostasis is already significantly disrupted. Second, cirrhosis-related hepatic and extrahepatic problems may overlap with or intensify symptoms caused by IrAEs, hampered early and rapid detection, which is compulsory concerning the consequence of potentially life-threatening episodes. Thus, before starting ICI therapy, individuals with HCC should be carefully selected and evaluated. In general, IrAE management is built on three pillars. First, close monitoring is required, including weekly clinical controls and, depending on the severity of the incidents, hospitalization. Importantly, individuals with severe IrAEs should be referred to a specialized center as soon as possible. This is especially important in patients with liver cirrhosis because distinguishing between cirrhosis-associated complications and IrAEs can be difficult, and premature discontinuation of an effective antitumor therapy or starting of steroid therapy in cirrhotic patients can have serious consequences[160]. Second, depending on the type and severity of IrAEs, ICI therapy may need to be either interrupted or permanently discontinued. Apart from PD-1/PD-L1-driven rash, adrenal insufficiency, nephritis and hypothyroidism, which recover after 1 mo of cessation, permanent termination of ICI therapy should be considered for higher grade IrAEs[160]. Re-exposure to ICI therapy after withdrawal, on the other hand, is associated with a significant risk of IrAE recurrence

[161]. Third, for IrAEs of greater than grade 2, glucocorticoids (0.5 to 2 mg/kg/d prednisone oral or intravenous depending on the type and severity of IrAEs) may be needed. Cutaneous IrAEs are treated with topical, oral, or intravenous glucocorticoids and topical or oral antihistamines, depending on the severity of clinical presentation. Cutaneous IrAEs range from commonly observed rash or pruritus to very less common but more severe disorders such as Stevens-Johnson syndrome[160]. Steroids should be continued for at least 3 d and then tapered over a period of 1 wk to 4 wk[162,163].

Differential diagnosis is required for gastrointestinal IrAEs, notably colitis and/or diarrhea, in order to rule out infectious disorders and pharmacological side effects[160]. In the event of glucocorticoid failure, immunosuppressive medication (such as vedolizumab or infliximab) should be started as soon as possible[164]. The diagnosis and management of immune-related hepatitis in HCC patients receiving ICI therapy is particularly difficult[160]. As a result, seeking the advice of an experienced hepatologist as soon as possible is strongly advised. Intrahepatic tumor growth, hepatitis B virus and/or hepatitis C virus or newly acquired viral hepatitis, cytomegalovirus reactivation, hepatotoxic medication side effects, cholestasis, and ascites should all be ruled out before diagnosing immune-related hepatitis[160]. A liver biopsy should also be considered prior to steroid therapy. For severe cases of immune-related hepatitis, oral or intravenous steroids may be used[160]. After the toxicity has subsided, tapering should be done over a period of 4-6 wk[162,163].

Pneumonitis is a potentially life-threatening IrAE. As a result, the pneumonitis should be followed by a prompt and thorough differential diagnosis, including the elimination of viral etiologies, hepatopulmonary syndrome and porto-pulmonary hypertension[160]. Steroids should be started, and tapering should take 4-6 wk[163]. Following glucocorticoid failure, infliximab or mycophenolate mofetil may be administered[160]. Thyroid-related IrAEs include hypothyroidism and hyperthyroidism caused by thyroiditis. A progressive reduction in thyroid-stimulating hormone in conjunction with normal or decreasing thyroxine levels should trigger repeated cortisol assessments to rule out immune-related hypopituitarism. In the case of hypothyroidism, thyroxine substitution is only recommended in symptomatic patients[162]. Thyroid antibodies and uptake should be assessed in symptomatic hyperthyroid patients, and beta-blockers and/or carbimazole should be investigated. Asymptomatic patients do not require any special treatment, and ICI therapy should be continued. Patients on ICI therapy should have regular thyroid stimulating hormone and free thyroxine tests. If central hypothyroidism is suspected, each pituitary hormone axis should be examined[160]. Cortisol, corticotropin-releasing hormone, adrenocorticotropic hormone, luteinizing hormone, thyroid stimulating hormone, free thyroxine, follicle-stimulating hormone, estradiol (premenopausal women), testosterone (men), insulin-like growth factor 1, and electrolytes are examples of these hormones[162,163].

CONCLUSION

Finally, HCC is a multifaceted illness with multiple faces. It avoids early discovery, which provides the best chance of cure by resection/transplant, and systemic treatments are only of marginal efficacy at best, despite recent therapeutic advances. Current advances in immunotherapy and its combinations have altered the HCC treatment landscape and clinical studies are continuing to pave the path forward. Immunotherapy increases survival rates and provides long-term cancer control in subsets of HCC patients while also minimizing side effects. Further research into immunotherapy in combination with current treatments for HCC in the early and intermediate stages may assist a greater spectrum of patients. Continued research into PD-1/PD-L1, TMB, ctDNA, microsatellite stability, DNA mismatch repair, neutrophil/lymphocyte ratio, cytokines, and cellular peripheral immune response will hopefully identify the most reliable marker for selecting and sequencing systemic treatments to achieve the best outcome in HCC patients. Despite such significant treatment advances in HCC, numerous hurdles remain. The scientific community must figure out how to appropriately sequence these medicines for the best potential response, how to control toxicities, and how to develop indicators to monitor for response and relapse.

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ORIGINAL ARTICLE

Retrospective Study

Clinical-radiomics nomogram for predicting esophagogastric variceal bleeding risk noninvasively in patients with cirrhosis

Rui Luo, Jian Gao, Wei Gan, Wei-Bo Xie

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Rui Luo, Jian Gao, Department of Gastroenterology and Hepatology, The Second Affiliated Hospital of Chongqing Medical University, Chongqing 400010, Chongqing, China

Wei Gan, Wei-Bo Xie, Department of Radiology, The Second Affiliated Hospital of Chongqing Medical University, Chongqing 400010, Chongqing, China

Corresponding author: Jian Gao, MMed, PhD, Chief Doctor, Professor, Department of Gastroenterology and Hepatology, The Second Affiliated Hospital of Chongqing Medical University, No. 76 Linjiang Road, Yuzhong District, Chongqing 400010, Chongqing, China. gaojian@cqmu.edu.cn

Abstract

BACKGROUND

Esophagogastric variceal bleeding (EGVB) is a serious complication of patients with decompensated cirrhosis and is associated with high mortality and morbidity. Early diagnosis and screening of cirrhotic patients at risk for EGVB is crucial. Currently, there is a lack of noninvasive predictive models widely available in clinical practice.

AIM

To develop a nomogram based on clinical variables and radiomics to facilitate the noninvasive prediction of EGVB in cirrhotic patients.

METHODS

A total of 211 cirrhotic patients hospitalized between September 2017 and December 2021 were included in this retrospective study. Patients were divided into training (n = 149) and validation (n = 62) groups at a 7:3 ratio. Participants underwent three-phase computed tomography (CT) scans before endoscopy, and radiomic features were extracted from portal venous phase CT images. The independent sample t-test and least absolute shrinkage and selection operator logistic regression were used to screen out the best features and establish a radiomics signature (RadScore). Univariate and multivariate analyses were performed to determine the independent predictors of EGVB in clinical settings. A noninvasive predictive nomogram for the risk of EGVB was built using independent clinical predictors and RadScore. Receiver operating characteristic, calibration, clinical decision, and clinical impact curves were applied to evaluate the model's performance.

RESULTS

Albumin (P = 0.001), fibrinogen (P = 0.001), portal vein thrombosis (P = 0.002), aspartate aminotransferase (P = 0.001), and spleen thickness (P = 0.025) were selected as independent clinical predictors of EGVB. RadScore, constructed with five CT features of the liver region and three of the spleen regions, performed well in training (area under the receiver operating characteristic curve (AUC) = 0.817) as well as in validation (AUC = 0.741) cohorts. There was excellent predictive performance in both the training and validation cohorts for the clinical-radiomics model (AUC = 0.925 and 0.912, respectively). Compared with the existing noninvasive models such as ratio of aspartate aminotransferase to platelets and Fibrosis-4 scores, our combined model had better predictive accuracy with the Delong's test less than 0.05. The Nomogram had a good fit in the calibration curve (P > 0.05), and the clinical decision curve further supported its clinical utility.

CONCLUSION

We designed and validated a clinical-radiomics nomogram able to noninvasively predict whether cirrhotic patients will develop EGVB, thus facilitating early diagnosis and treatment.

Key Words: Liver cirrhosis; Variceal bleeding; Radiomics; Nomogram; Diagnosis

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Core Tip: Esophagogastric variceal bleeding (EGVB) is a life-threatening complication of liver cirrhosis. Currently, no noninvasive prediction models for bleeding risk are widely used in clinical practice. Our study extracted radiomics features from computed tomography and identified portal vein thrombosis, fibrinogen, aspartate aminotransferase, albumin, and spleen thickness as independent clinical predictors. Consequently, we established a novel clinical-radiomics model for noninvasive prediction of EGVB. This model exhibits superior diagnostic performance and can assess bleeding risk early. It has the potential to facilitate early prevention and treatment of possible bleeding in cirrhotic patients with esophagogastric varices.

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INTRODUCTION

In liver cirrhosis, esophagogastric variceal bleeding (EGVB) is among the most common complications associated with portal hypertension, characterized by acute life-threatening sequelae and high mortality rates[1]. Approximately 50% of patients with cirrhosis develop esophagogastric varices (EGV) and approximately 10%–15% of these rupture and hemorrhage annually. Almost 12% – 20% of patients die within 6 wk of acute variceal bleeding, and 60% are likely to rebleed within a year, thus endangering their lives and degrading their quality of life[1-3]. Therefore, the early identification of cirrhotic patients at risk of EGVB is essential.

Endoscopy is the gold standard for detecting patients with EGVB, which is recommended by guidelines for diagnosing and screening EGV as well as for assessing bleeding risk[4]. However, some patients refuse upper gastrointestinal endoscopy due to its invasiveness and poor tolerance. Furthermore, repeated examinations may reduce long-term compliance and thereby confound clinical observation. Moreover, endoscopy has the potential to provoke EGVB in low-risk patients, and carries the attendant risks of anesthesia. Consequently, regular follow-up through endoscopy is difficult.

The Baveno VII consensus workshop recommended that patients with compensated cirrhosis whose liver stiffness and platelet counts meet cut-off points may be spared endoscopic screening[3]. However, because the Baveno criteria have a low specificity for ruling out high-risk varices, some patients still require a subsequent endoscopy[5]. Ultrasound elastography is impeded by ascites, obesity, bowel gas, and other clinical issues. The above reasons limit the application of the Baveno criteria in clinical practice. Measurement of the hepatic venous pressure gradient serves as the gold standard for the diagnosis of portal hypertension and to assess treatment response, which is an effective tool for predicting the risk of bleeding at the EGV[3]. However, its measurement entails an invasive procedure that requires specialized operators and equipment, as well as high economic costs that limit its availability in clinical practice.

Conversely, the widespread use of computed tomography (CT) in cirrhotic patients facilitates the diagnosis and evaluation of complications. Typical CT manifestations of cirrhosis include disproportionate sizes of hepatic lobes, marked widening of hepatic fissures, wavy patterns of hepatic edges, visible gallbladder displacement, and heterogenous hepatic parenchymal density[6]. In addition, CT images can visualize cirrhosis-related complications such as portal vein widening, portal hypertension, collateral circulation, and splenomegaly[7]. All of these manifestations can facilitate the diagnosis of cirrhosis. Nonetheless, the utility of CT-based screening depends highly on the experience and subjective judgement of the radiologist.

Recent studies on the noninvasive assessment and prediction of EGV have made progress. Radiomics is an emerging image analysis technology that focuses on extracting high-throughput and quantitative features from medical images, using data mining algorithms to detect associations between radiographic features and diseases, and to then develop corresponding models for each feature[8]. It is used primarily for tumor diagnosis, efficacy evaluation, and prognosis[9]. Only a few studies have utilized it to assess non-neoplastic liver diseases and portal hypertension[10-14]. The CHESS team from China established the first model for predicting clinically significant portal hypertension based on radiomics features of liver and spleen, and demonstrated good diagnostic performance (area under the receiver operating characteristic curve (AUC) = 0.894)[11]. Additionally, Lin *et al*[15] evaluated the safety and efficacy of predicting high-risk EGV by combining esophageal and gastric radiomics features. By extracting CT features from hepatic, splenic, and lower esophageal regions of interest (ROIs), Liu *et al*[12] combined radiomics signature with clinical indicators to establish a prediction model to identify the likelihood esophageal variceal bleeding. Yang *et al*[14] integrated radiomics from the liver region with clinical variables to estimate the bleeding risk of EGV in cirrhosis related to hepatitis B.

However, previous radiomics models have not explored the risk of EGVB in patients with cirrhosis of varied etiologies. Furthermore, their performance has not been compared to existing noninvasive models. The establishment of a noninvasive prediction model may reduce the frequency of endoscopic examinations in patients without EGVB, while patients at risk of EGVB may receive more timely diagnosis and treatment, which is of great significance for guidance of follow-up treatment and review. Therefore, our study sought to develop a noninvasive predictive model that combines radiomics features of the liver and spleen as well as clinical indicators for predicting the risk of EGVB in patients with cirrhosis, and to evaluate its clinical utility.

MATERIALS AND METHODS

This was a retrospective study conducted at a single center, with an informed consent waiver granted by the Institutional Review Board.

Patients

Patients diagnosed with liver cirrhosis and EGV between September 2017 and December 2021 were included in our study by retrospective review. The following were the criteria for inclusion: (1) Diagnosis of cirrhosis through histopathologic examination or a combination of clinical, laboratory, and radiologic findings; (2) Completion of a triple-phase enhanced CT scan within one week prior to endoscopy; and (3) All patients diagnosed with EGV through endoscopy. Exclusion criteria were: (1) Previous endoscopic therapy, trans jugular intrahepatic portosystemic shunt, splenectomy, splenic artery embolization, balloon occlusion, retrograde transvenous occlusion, or liver transplantation; (2) Patients with liver neoplasms; and (3) Incomplete clinical or imaging data.

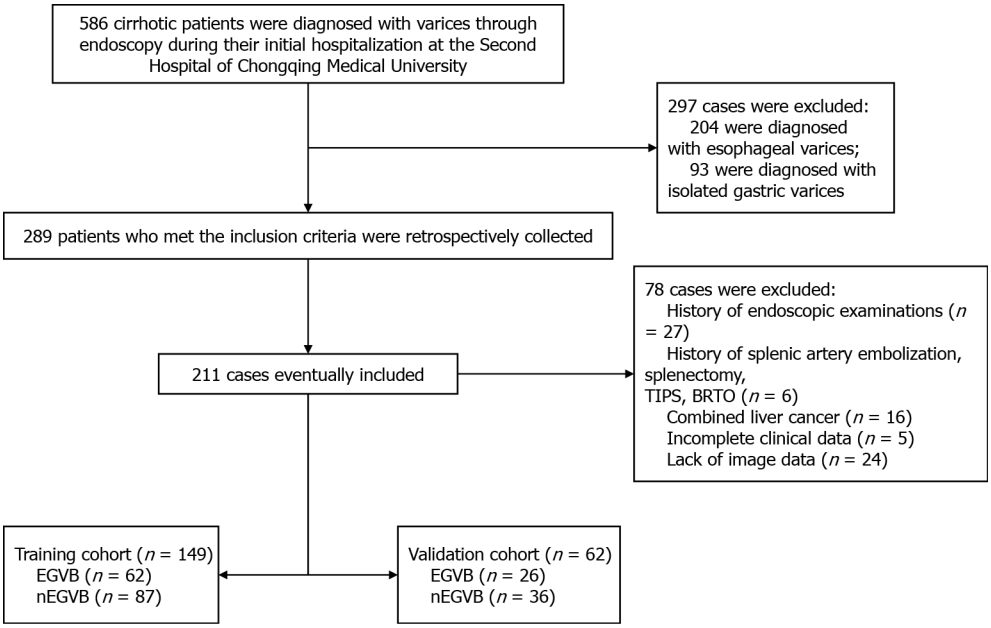
As a random assignment in a 7:3 ratio, patients were divided into training and validation cohorts. Finally, there were 149 patients included in the training set, as well as 62 in the validation one. A flowchart of our study is shown in Figure 1.

Endoscopy

Endoscopies were performed by experienced gastroenterologists. The diagnosis of EGVB-positive was based on one or more of the following signs: (1) Bleeding or exudation of the variceal region; (2) Fresh blood, with the exclusion of non-variceal hemorrhage; and (3) Signs of recent variceal bleeding (stain), such as a white thrombus or an overlying blood clot[4,16]. Ulcers, portal hypertensive gastropathy, and other non-variceal factors may also lead to upper gastrointestinal bleeding. When patients had a recent history of bleeding and endoscopy failed to find evidence of variceal hemorrhage, we considered other etiologies of bleeding such as portal hypertensive gastropathy and ulcers.

CT Scan

CT examinations were performed using a Siemens SOMATOM Force dual-source CT scanner (Siemens Healthcare). Patients were instructed to fast for more than four hours without consuming any solid food or liquids. They were allowed to drink up to 800 mL of water before scanning. Iohexol (350 mg/mL) was injected under high pressure into the cubital vein at a dose of 1.5 mL/kg and at a flow rate of 4.5 mL/s, followed by flushing with 30 mL of a saline solution. Siemens CT used contrast agent concen-



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Figure 1 Flowchart of inclusion and exclusion procedures for this study. TIPS: Transjugular intrahepatic portosystemic shunt; BRTO: Balloon occluded retrograde transvenous obliteration; EGVB: Esophagogastric variceal bleeding; nEGVB: Without the occurrence of esophagogastric variceal bleeding.

tration tracking technology to obtain standard three-phase images of the abdominal aorta after setting a threshold of 100HU and triggering delays of 10 s, 20 s, and 15 s. Scan parameters were: tube voltage, 120 kV or 70 kV; tube current, 148 mAs; slice thickness, 1 mm; slice interval, 0 mm; and pitch ratio, 0.6. Feature extraction was performed on all images and exported in Digital Imaging and Communications in Medicine format.

Measurements of related parameters from CT images[17] were: (1) Portal vein thrombosis: endovascular filling defect of the portal vein; (2) Splenorenal/gastrorenal shunts: tortuous and thick vessels between the lesser curvature of the stomach, the splenic hilum, and left renal hilus, which may dilate and communicate with the left renal vein; (3) Upper and lower splenic diameters: The vertical distance from the upper to the lower margin of the spleen, *i.e.*, the lower pole window minus the upper pole window of the spleen; (4) Spleen diameter: The longest diameter of the spleen at the central level of the splenic hilum (antero-posterior straight line); and (5) Spleen thickness: The shortest diameter from the inner margin to the outer margin of the spleen at the central level of the splenic hilum.

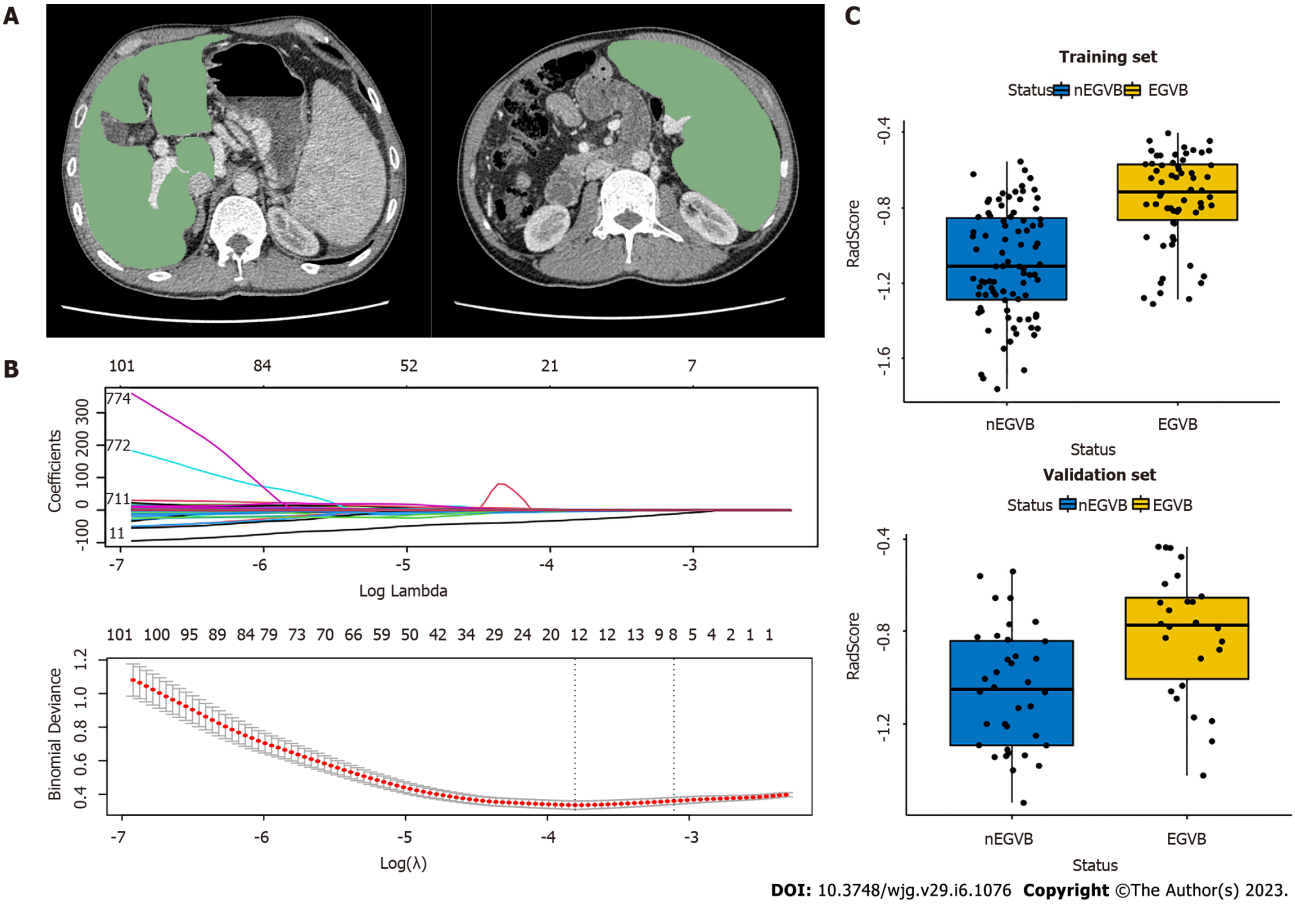
CT image preprocessing and ROI segmentation

After retrieving CT images from all patients, the radiologist used the 3Dslicer (V4.13.0, https://www.slicer.org/) to delineate ROI in the portal venous phase. ROIs of the liver and spleen were set at the levels of the hepatic and splenic hila, respectively (Figure 2A), excluding the large blood vessels. The radiologist was blinded to the endoscopic results.

Feature extraction and selection in radiomics

Before the extraction of radiomic features, images were normalized by Gaussian filtering, wavelet bandpass filtering, and isotropic resampling. Based on ROIs, we could extract radiomics features through the Pyradiomics-based extension package Radiomics of 3Dslicer. There were 1130 radiomic features extracted for each ROI, totaling 2260 per patient.

To analyze inter- and intra-observer reliability, 30 cases were selected randomly from the total sample. After two months, two radiologists experienced in abdominal CT interpretation repeated segmentation and feature extraction for these cases. Reliability was assessed by the intraclass correlation coefficient (ICC), and was considered strongly reproducible and dependable when ICC values exceeded 0.75. Features above the ICC threshold of 0.75 were included in subsequent studies. To reduce the overfitting caused by a great number of redundant features, an independent sample t-test was applied to features and classification labels to screen out highly correlated features. Next, the least absolute shrinkage and selection operator (LASSO) regression was conducted to obtain dimensionality reduction, retain the most relevant features, and construct radiomic features from non-zero coefficient features within one standard deviation of the optimal penalty parameter lambda. A radiomics signature (RadScore) was calculated and constructed for each patient through a linear combination of selected features weighted by their respective LASSO coefficients.



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Figure 2 Workflow of the radiomics analysis. A: The liver and spleen regions of interest were defined at the level of the hepatic hilum and the splenic hilum, respectively; B: The least absolute shrinkage and selection operator regression analysis for dimension reduction of radiomics features, and the optimal model parameter λ was selected by 10-fold cross-validation; C: The boxplots of radiomics signatures (RadScore) in the training and validation groups display notable differences respectively. EGVB: Esophagogastric variceal bleeding; nEGVB: Without the occurrence of esophagogastric variceal bleeding.

Statistical analysis

Quantitative data from normal distribution are represented by X (standard deviation), from non-normal distribution by M (P25-P75), and count data by percentage. Both univariate logistic and multiple logistic regressions with backward stepwise selection were used for the selection of independent risk factors for clinical variables. For the training and validation cohorts, we calculated the AUC the receiver operating characteristic curves to evaluate the predictive accuracy of clinical-radiomic models. Furthermore, the clinical-radiomics nomogram was subjected to decision curve and clinical impact curve analyses to determine its clinical utility and net benefit. There were two statistical analysis software programs carried out for this study: R (version 4.1.3) and Python (version 3.9.7). Statistical tests were all two-way, and it was considered statistically significant when the *P*-values were less than 0.05.

RESULTS

Patient characteristics

In our study, there were a total of 211 patients diagnosed with cirrhosis and EGV (131 men and 80 women), 123 of whom were identified as EGVB-negative (nEGVB) and 88 were EGVB-positive. Thirteen were diagnosed with cirrhosis by histological examination, and the remaining 198 patients were diagnosed with cirrhosis by a combination of history, clinical manifestations, laboratory findings, and CT examination. Of these, some were hospitalized for acute decompensation events such as bleeding, ascites, and hepatic encephalopathy in a short-term period. Hemorrhage occurred primarily in the EGVB-positive group, while ascites and hepatic encephalopathy were present in both EGVB-positive and -negative groups.

From electronic medical record systems, we obtained the complete demographic and clinical data about patients. Table 1 summarizes the baseline characteristics of patients.

Table 1 Baseline characteristics of patients in the training and validation cohort

Characteristics	Train cohort		Validation cohort	
	nEGVB (*n* = 87)	EGVB (*n* = 62)	nEGVB (*n* = 36)	EGVB (*n* = 26)
Sex (Male)	54 (62.1%)	40 (64.5%)	21 (58.3%)	16 (61.5%)
Age(yr)	55.0 (36.0, 83.0)	53.0 (15.0, 79.0)	52.0 (10.8)	52.9 (12.5)
Etiology				
HBV	58 (66.7%)	38 (61.3%)	19 (52.8%)	16 (61.5%)
HCV	5 (5.7%)	6 (9.7%)	3 (8.3%)	1 (3.8%)
Alcohol	4 (4.6%)	7 (11.3%)	3 (8.3%)	3 (11.5%)
Others	20 (23.0%)	11 (17.7%)	11 (30.6%)	6 (23.1%)
Portal vein thrombosis	5 (5.7%)	21 (33.9%)	1 (2.8%)	8 (30.8%)
Shunt	21 (24.1%)	21 (33.9%)	14(38.9%)	9 (34.6%)
HE	1 (1.1%)	6 (9.7%)	0 (0%)	2 (7.7%)
Ascites				
0	40 (46.0%)	9 (14.5%)	13 (36.1%)	6 (23.1%)
1	31 (35.6%)	45 (72.6%)	22 (61.1%)	17 (65.4%)
2	5 (5.7%)	6 (9.7%)	1 (2.8%)	2 (7.7%)
3	11 (12.6%)	2 (3.2%)	0 (0%)	1 (3.8%)
Child-Pugh score				
A	33 (37.9%)	16 (25.8%)	15 (41.7%)	9 (34.6%)
B	47 (54.0%)	38 (61.3%)	17 (47.2%)	14 (53.8%)
C	7 (8.0%)	8 (12.9%)	4 (11.1%)	3 (11.5%)
PLT (10^9/L)	60.0 (17.0, 186)	72.5 (21.0, 245)	53.0 (16.0, 192)	60.0 (26.0, 208)
PT (s)	15.6 (12.4, 24.9)	16.1 (11.7, 26.8)	16.1 (3.30, 22.3)	17.1 (14.1, 31.6)
APTT (s)	39.7 (29.5, 57.3)	38.9 (30.1, 53.1)	41.5 (34.2, 51.0)	38.2 (30.7, 63.6)
INR	1.22 (0.92, 2.18)	1.28 (0.85, 2.51)	1.29 (0.98, 1.95)	1.39 (1.10, 3.00)
Fib (g/L)	2.19 (0.75, 6.09)	1.78 (0.60, 5.78)	2.11 (0.489)	1.80 (0.628)
Albumin (g/L)	34.1 (18.4, 46.3)	32.3 (21.3, 45.9)	33.6 (6.36)	30.8 (5.28)
Tbil (μmol/L)	21.9 (2.20, 366)	16.9 (3.60, 93.0)	23.5 (6.40, 414)	18.5 (7.00, 76.1)
DBil (μmol /L)	9.80 (0.90, 276)	8.05 (1.90, 65.9)	10.8 (2.30, 306)	8.70 (2.70, 38.5)
Prealbumin (mg/L)	90.0 (12.0, 220)	97.5 (18.0, 246)	85.0 (9.00, 268)	83.5 (18.0, 221)
ALT (U/L)	36.0 (7.00, 345)	23.0 (10.0, 85.0)	41.0 (14.0, 337)	22.5 (10.0, 107)
AST (U/L)	47.0 (15.0, 330)	29.5 (11.0, 92.0)	48.0 (23.0, 319)	25.5 (17.0, 121)
SL (mm)	130 (89.1, 206)	142 (78.8, 200)	143 (98.7, 199)	139 (109, 205)
SD (mm)	144 (82.0, 235)	157 (68.8, 299)	152 (86.0, 239)	174 (109, 222)
ST (mm)	45.1 (9.22)	52.5 (11.1)	48.3 (30.5, 84.5)	55.2 (29.9, 64.8)

EGVB: Esophagogastric variceal bleeding; nEGVB: Without the occurrence of esophagogastric variceal bleeding; HE: Hepatic encephalopathy; PLT: Platelet. PT: Prothrombin time; APTT: Activated partial thromboplastin time; INR: International prothrombin ratio; Fib: Fibrinogen; Tbil: Total bilirubin; DBil: Direct bilirubin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; SL: Spleen upper and lower diameter; SD: Spleen diameter; ST: Spleen thickness.

Radiomics Feature Selection and Radiomics Signature Construction

Based on an independent sample t-test and LASSO regression, eight radiomics features were selected, five from the liver and three from the spleen (Figure 2B). Through weighting the LASSO coefficients of the selected features, we established a radiomics signature (RadScore). The specific formula of RadScore

is shown in the supplementary material. The boxplots in the training and validation cohorts indicated that RadScores differed remarkably ($P < 0.001$) (Figure 2C).

Model Construction and Evaluation

Univariate analysis screened out clinical features that were significantly related to EGVB ($P < 0.05$). To minimize the effects of multicollinearity, we performed a multivariate backward stepwise regression to confirm portal vein thrombosis, aspartate aminotransferase (AST), albumin, fibrinogen, and spleen thickness as independent risk factors for EGVB ($P < 0.05$) (Table 2). The variance inflation factor was less than 4.

By combining independent clinical predictors with RadScore, a clinical-radiomics nomogram was established by generalized linear regression (Figure 3). The calibration curve performed well in both training and validation groups, with Hosmer-Lemeshow test results of $P = 0.775$ (Figure 4A) and $P = 0.391$ (Figure 4B) respectively, indicating that the nomogram's predictive power and the actual risk of developing EGVB did not differ significantly. As shown in the training set (Figure 5A), the clinical-radiomics nomogram demonstrated excellent predictive performance with an AUC of 0.925 (95%CI: 0.883-0.967) and 0.912 (95%CI: 0.845-0.98) as shown in the validation set (Figure 5B). Furthermore, compared with other noninvasive methods such as APRI and FIB-4 reported in studies previously, our clinical-radiomics nomogram exhibited the highest diagnostic accuracy, with Delong test of less than 0.05. Decision curve analysis showed that the clinical-radiomics nomogram had a high clinical applicability that exceeded those of existing noninvasive serum-based predictive models such as APRI and FIB-4 in both the training (Figure 6A) and validation (Figure 6B) sets. Moreover, the clinical impact curve of the nomogram was constructed based on 1,000 simulated samples, which revealed that the nomogram yielded an event probability that corresponded closely to actual high risk when the risk threshold was between 0.4 and 0.8, and consistently when it exceeded 0.8 (Figure 7).

DISCUSSION

With a high mortality and morbidity rate, EGVB is among the most serious complications related to decompensated cirrhosis[1]. A routine endoscopic examination is recommended as a diagnostic method for assessing the grade and bleeding risk of EGV[4]. However, the bleeding risk of invasive procedures, contraindications to anesthesia, and unnecessarily excessive endoscopic examinations limit its clinical application and, to some extent, lead patients to forego their follow-ups. In recent years, there has been an increasing demand for noninvasive methods to estimate the risk of EGVB in cirrhotic patients. Noninvasive diagnostic methods such as ultrasonography, magnetic resonance imaging, and CT have both advantages and disadvantages[18-20], and there are no standardized noninvasive models for EGVB that can be used widely in clinical practice.

Radiomics is an emerging diagnostic tool that has the ability to extract numerous image features noninvasively and describe modules for quantitatively evaluating gray scales and pixel distribution in medical images, which are undetectable by the naked eye[21]. It has yielded satisfactory results, and has become increasingly popular in diagnosis, assessment of treatment response, and prognosis in recent years[8]. It can comprehensively describe ROIs from the perspectives of morphology, histogram, texture, and higher-order features such as texture features extracted through Gaussian, wavelet, and other filtration methods[22].

We constructed a RadScore based on five liver features and three spleen features. Among them, 5 texture and 3 histogram features were extracted, 7 of which were higher-order filtered features. Small Area Low Gray Level Emphasis, Size Zone Non Uniformity Normalized, Short Run High Gray Level Emphasis, Cluster Shade, and Imc1 are texture features[23]. As crucial components of radiomics features, they combine pixels and shapes, and thereby describe the spatial relationships of adjacent pixels. Kurtosis, Skewness and Maximum are histogram features that emphasize the distribution pattern of gray-level pixel values within ROIs[23]. RadScore can accurately predict EGVB risk as evidenced AUCs of both the training and validation sets, and performed better in the validation group in comparison to Yang *et al*[14]'s previous study that only included liver ROIs.

There is no single radiomics model which is capable of fully reflecting the characteristics of the disease. Consequently, multiparametric, multi-omics, and multivariate models have generated increasing interest. By incorporating readily available independent clinical predictors and RadScore, diagnostic performance has been further enhanced, with an AUC of 0.925 (95%CI: 0.883-0.967) and 0.912 (95%CI: 0.845-0.98) in the training and validation sets, respectively. Meanwhile, our combined model performed better in predicting EGVB than other noninvasive methods (such as APRI and FIB-4) that have been fully validated in previous studies. Decision curve analysis showed that the clinical-radiomics nomogram can yield a good net benefit in clinical applications. The reduced AUCs of all models in the validation cohort are readily apparent, and may have been caused by the small sample size. In addition, the EGVB group included 29 patients with variceal bleeding at the esophagogastric junction and 27 at the gastric fundus. To some extent, our model could predict the overall risk of variceal bleeding in the esophagogastric junction and gastric fundus. However, no radiomics-related

Table 2 Univariate and multivariate analysis of factors associated with the presence of esophagogastric variceal bleeding in cirrhotic patients

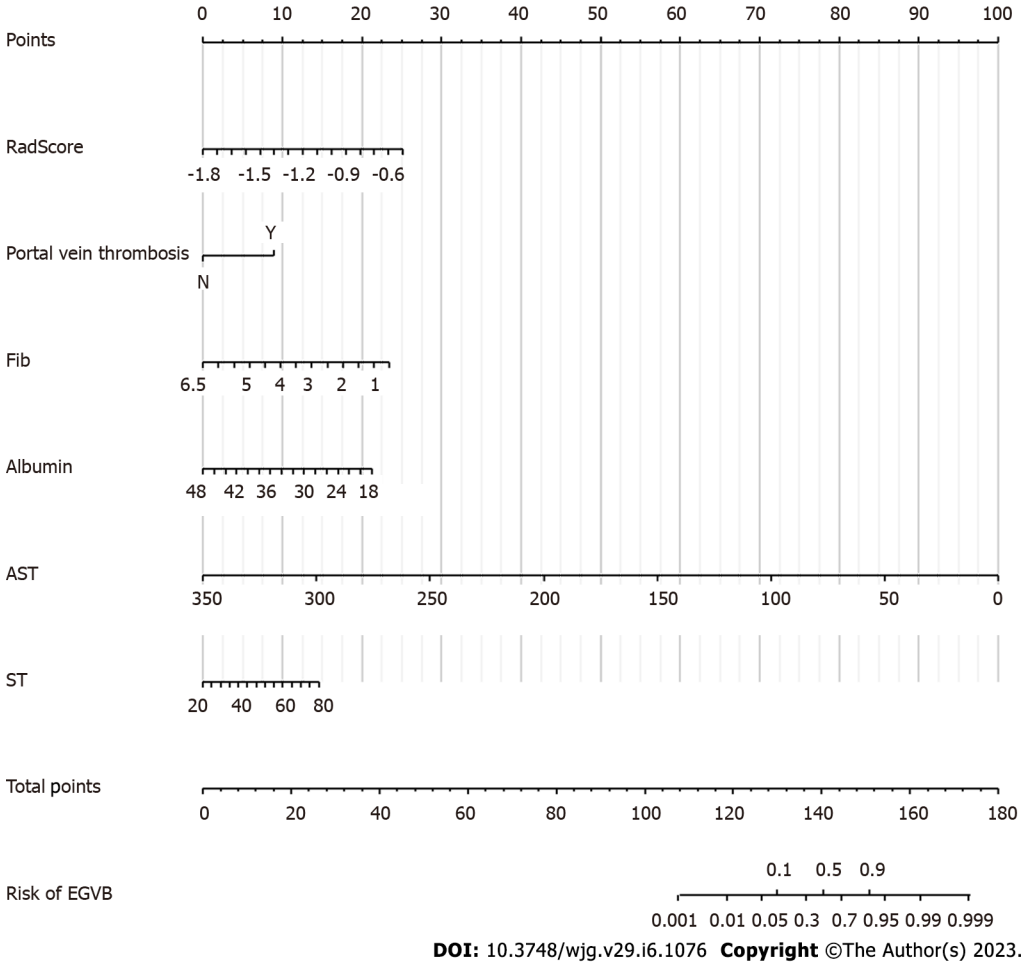
Variables	Univariate logistic regression		Multivariate logistic egression	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Age	0.98 (0.949-1.01)	0.183	-	-
Albumin	0.925 (0.866-0.984)	0.01	0.84 (0.753-0.926)	< 0.001
ALT	0.962 (0.941-0.984)	0.001	-	-
APTT	0.945 (0.876-1.01)	0.107	-	-
AST	0.962 (0.942-0.981)	< 0.001	0.941 (0.913-0.97)	< 0.001
DBil	0.968 (0.926-1.01)	0.122	-	-
Etiology	1.832 (-0.094-3.76)	0.661	-	-
Fib	0.46 (-0.061-0.982)	0.004	0.385 (-0.17-0.939)	0.001
INR	5.613 (4.26-6.97)	0.013	-	-
PLT	1.007 (0.998-1.02)	0.11	-	-
Portal vein thrombosis	8.4 (7.36-9.44)	< 0.001	9.15 (7.78-10.5)	0.002
HE	9.214 (7.07-11.4)	0.042	-	-
Child-Pugh score	1.668 (1.07-2.26)	1.365	-	-
Ascites	6.452 (6.87-6.03)	0.853	-	-
Prealbumin	1.002 (0.995-1.01)	0.525	-	-
PT	1.188 (1.05-1.33)	0.015	-	-
SD	1.01 (1-1.02)	0.016	-	-
Sex	0.9 (0.223-1.58)	0.76	-	-
Shunt	1.61 (0.89-2.33)	0.195	-	-
SL	1.012 (0.999-1.02)	0.075	-	-
ST	1.078 (1.04-1.12)	< 0.001	1.056 (1.01-1.1)	0.025
Tbil	0.979 (0.955-1)	0.08	-	-

HE: Hepatic encephalopathy; PLT: Platelet; PT: Prothrombin time; APTT: Activated partial thromboplastin time; INR: International prothrombin ratio; Fib: Fibrinogen; Tbil: Total bilirubin; DBil: Direct bilirubin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; SL: Spleen upper and lower diameter; SD: Spleen diameter; ST: Spleen thickness.

studies have specifically addressed the risk of variceal bleeding at these two anatomic sites. Prospective exploration and analysis in follow-up studies are necessary.

Cirrhosis features fibrosis and regenerative hepatic nodules that compress sinusoids, resulting in sinusoidal narrowing that promotes vascular collateralization[1]. Furthermore, portal hypertension predisposes to branched splenic vein obstruction, leading to congestive splenomegaly and hypersplenism. Numerous studies have confirmed associations between liver, spleen, and portal hypertension [18,24]. In earlier studies of radiomics models for predicting variceal bleeding in cirrhosis, Liu's research only examined esophageal variceal bleeding[12], and Yang *et al*'s study only included liver ROIs[14]. In contrast to previous reports, we included patients with cirrhosis of multiple etiologies and extracted hepatic and splenic features simultaneously. Additionally, our combined model showed a better performance in comparison with other multivariate ones.

Our study found that portal vein thrombosis, fibrinogen, AST, albumin, and spleen thickness were independent risk factors for EGVB in patients with cirrhosis. According to previous studies, portal vein thrombosis is one of the risk factors for EGVB in liver cirrhosis, which may be related to the increased portal pressure to some extent[25,26]. Impaired hepatic synthetic capacity disturbs procoagulant (*e.g.*, fibrinogen) and anticoagulant balance, resulting in coagulopathies. The decrease of fibrinogen may result in an inefficient removal of activated coagulation factors and ultimately increase the risk of bleeding. AST is a functional hepatic enzyme that can indirectly reflect hepatocellular injury. Albumin and spleen thickness are significantly correlated with the presence of EGV, and were considered as risk factors in previous research[27-29]. In contrast to several previous studies, we found that platelet count was not an independent clinical predictor and may be related to its predisposing factors such as hypers-

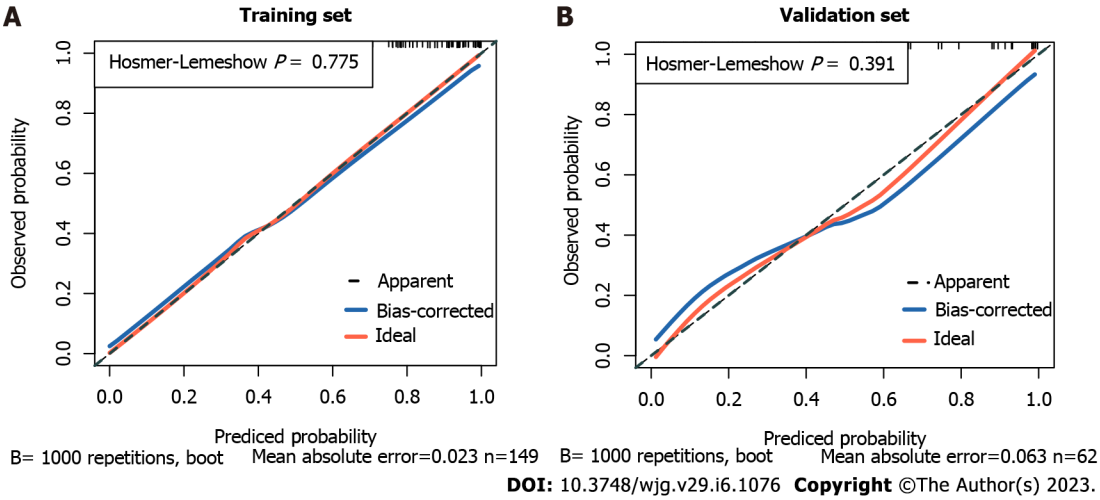


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Figure 3 The construction of a clinical-radiomics prediction model for esophagogastric variceal bleeding. The clinical-radiomics nomogram for predicting bleeding risk. The scores of the different variables in the nomogram were summed to obtain the total score, and a line was drawn from the axis of the total score to the bottom line to determine the probability of bleeding risk. Fib: Fibrinogen; AST: Aspartate aminotransferase; ST: Spleen thickness; EGVB: Esophagogastric variceal bleeding.

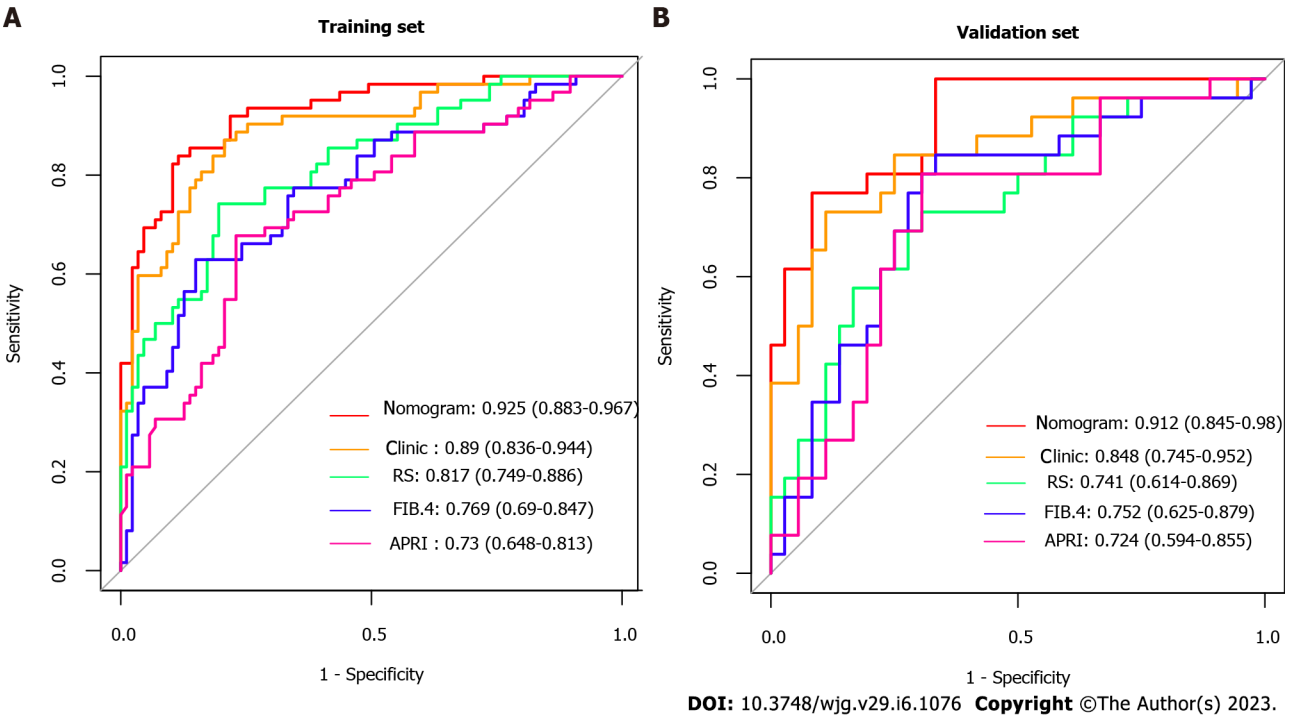
plenism, antibody-mediated destruction (primarily during viral hepatitis), reductions in thrombopoietin, and bone marrow toxicity caused by hepatitis viruses and alcohol. The Child Pugh score can reflect hepatic functional reserve, often as a prognostic indicator in patients with cirrhosis. Most of the patients in our retrospective study had splenomegaly and hypersplenism, and Child Pugh scores were predominantly grade B in both the EGVB and nEGVB groups. Child Pugh scores, platelet counts, and total bilirubin levels were similar between the two groups ($P > 0.05$). Similar results were also shown previously[30,31].

Several limitations have been identified in our study. Firstly, this is a small-scale single-center retrospective study. Large, multicenter and prospective studies will be required to verify the model's feasibility in the future. Moreover, there is no relevant study to explore the performance of models in predicting the risk of bleeding in patients with different Child Pugh class. Our study has demonstrated that there was no significant difference in the Child Pugh scores between the EGVB and nEGVB group, and the prediction model performed well in assessing the risk of bleeding on the whole. Exploring the diagnostic performance of the model for bleeding risk in patients with different Child Pugh class can facilitate further refining the risk stratification of patients and achieve precision medicine, which should be investigated in subsequent research. Secondly, elastography can predict portal hypertension and EGV to some extent by measuring liver and spleen stiffness. Lin *et al*[15] compared a radiomics nomogram with liver stiffness measurement through transient elastography in a subgroup of 42 patients, showing the nomogram was superior in predicting high-risk EGV. However, comparisons of elastography with radiomics models to predict variceal bleeding are lacking. In our retrospective study, most patients did not undergo elastography. We failed to retrieve the relevant data, which we see as a shortcoming of our study. The comparison of elastography with radiomics models is crucial, and should be conducted in the later studies. Thirdly, more in-depth investigations of multi-image and multi-omics (such as metabolomics, genomics, *etc*.) were not conducted. For subsequent studies of EGVB, multimodal and multi-omics models, deep learning, and other methods should be fully employed.



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Figure 4 The calibration curves of the nomogram. A: The calibration curve of the nomogram shows a good fit in the training cohort (P = 0.775); B: The calibration curve of the nomogram shows a good fit in the validation cohort (P = 0.391).

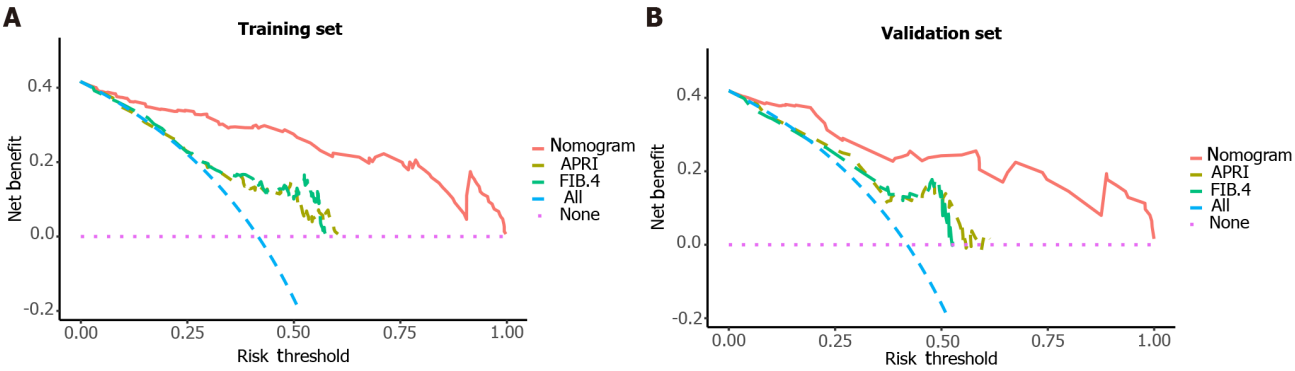


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Figure 5 The receiver operating characteristic curves in the study. A: The receiver operating characteristic curves (ROC) in the training cohort; B: The ROCs in the validation cohort. Clinic: The model based on clinical individual indicator; RS: Radiomics Signature (RadScore); APRI: Ratio of aspartate aminotransferase to platelets; FIB-4: Fibrosis-4 scores.

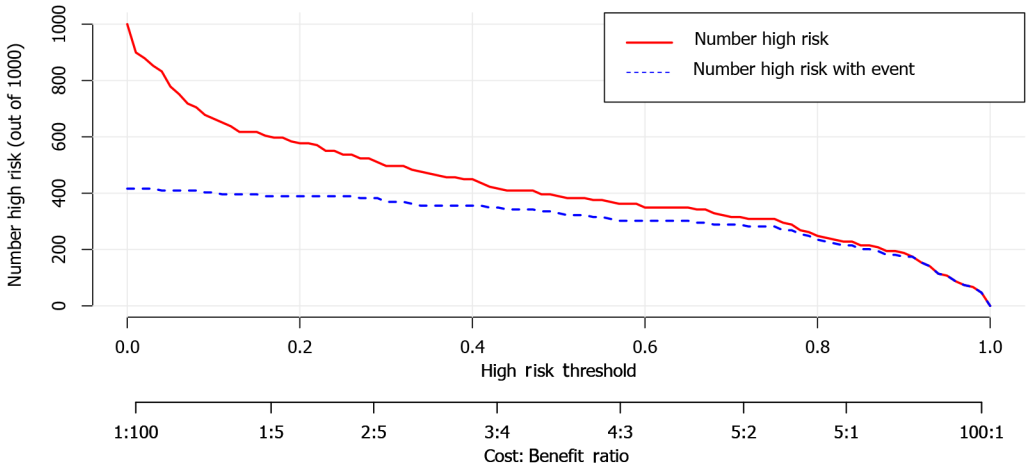
CONCLUSION

We established a multivariate prediction model based on CT radiomics features and clinical characteristics, which demonstrated good performance for predicting EGVB in cirrhotic patients. Aided by clinical-radiomics nomogram, clinicians can base therapeutic decisions on fewer invasive procedures while personalizing follow-up treatment.



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Figure 6 The clinical decision curves for bleeding prediction. A: The training cohort; B: The validation cohort. A threshold probability is the point at which the expected benefits of a treatment equal those of not receiving treatment. As shown by the different lines in the diagram, red indicates the clinical-radiomics nomogram, green indicates Fibrosis-4 scores (FIB-4), yellow indicates Ratio of aspartate aminotransferase to platelets (APRI), pink indicates the hypothesis of patients without esophagogastric variceal bleeding (EGVB), and blue indicates that all patients developed EGVB. They indicate that the clinical-radiomics nomogram is superior to the non-invasive serum predictive model's APRI and FIB-4 in terms of clinical utility. APRI: Ratio of aspartate aminotransferase to platelets; FIB-4: Fibrosis-4 scores.



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Figure 7 Clinical impact curve of the nomogram for 1,000 simulated samples. The blue dashed line is the actual high-risk number, and the red line is the high-risk number based on the nomogram. The nomogram predicted a probability of the event that corresponded closely to its actual high risk when the risk threshold was between 0.4 and 0.8, and consistently when it exceeded 0.8.

ARTICLE HIGHLIGHTS

Research background

Esophagogastric variceal bleeding (EGVB) is a fatal complication of liver cirrhosis, which requires aggressive intervention. Prediction of bleeding risk in cirrhotic patients with esophagogastric varices (EGV) is beneficial to individualized treatment and improve prognosis. Radiomics, an emerging field, has a good performance in disease diagnosis and efficacy evaluation.

Research motivation

Currently, there is still a lack of noninvasive models that can be widely used in clinical practice to predict the risk of bleeding in liver cirrhosis.

Research objectives

Our study aimed to develop and validate a novel predictive model based on radiomics extracted from contrast-enhanced computed tomography (CT) and clinical indicators to noninvasively assess the risk of bleeding in cirrhotic patients with EGV.

Research methods

211 patients were divided into training and validation cohorts in a 7:3 ratio. Radiomics features were extracted from the portal venous phase CT images, and a radiomics signature (RadScore) was constructed through further feature dimension reduction and screening. The univariate and multivariate logistic regression analyses were preformed to select independent clinical predictors. Finally, a combined model was established based on RadScore and clinical variables. The receiver operating characteristic curves, calibration curves, clinical decision curves and clinical impact curves were applied to evaluate the performance of the model.

Research results

The RadScore was constructed from 8 radiomics features. Albumin, fibrinogen, portal vein thrombosis, aspartate aminotransferase, and spleen thickness were selected as independent predictors. The nomogram, combining RadScore and clinical variables, demonstrated good diagnostic performance in both the training and validation cohorts (area under the receiver operating characteristic curve (AUC) = 0.925 and 0.912, respectively), which outperformed existing non-invasive models such as ratio of aspartate aminotransferase to platelets and Fibrosis-4 scores (Delong test < 0.05).

Research conclusions

The combined model based on radiomics features and clinical indicators shows good predictive accuracy and can contribute to noninvasively assessing the risk of EGVB in patients with cirrhosis.

Research perspectives

Radiomics has shown good diagnostic performance in the assessment of portal hypertension and the identification of high-risk esophageal varices. Our study demonstrated that the model combined clinical variables and radiomics features has the potential utility for non-invasive prediction of EGVB. Further large-scale, multi-center prospective studies are still required to verify its performance in the future.

FOOTNOTES

Author contributions: Luo R was responsible for designing the study, collecting data, analyzing data, and writing the paper; Gao J was responsible for designing the study and guiding important content of the article; Gan W and Xie WB were responsible for providing technical support. All authors approved the final version of the manuscript for submission for publication.

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Institutional review board statement: The study was reviewed and approved by the Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University (No. 2022-149).

Informed consent statement: Our study was a retrospective study conducted at a single center. We used data from patients at the time they were treated in the hospital, and these data were collected and analyzed anonymously. The informed consent waiver was granted by the Institutional Review Board.

Conflict-of-interest statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships.

Data sharing statement: No additional data are available.

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Country/Territory of origin: China

ORCID number: Rui Luo 0000-0002-5264-396X; Jian Gao 0000-0002-7761-4444.

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ORIGINAL ARTICLE

Observational Study

Survival benefit of younger gastric cancer patients in China and the United States: A comparative study

Peng-Hui Niu, Lu-Lu Zhao, Wan-Qing Wang, Xiao-Jie Zhang, Ze-Feng Li, Xiao-Yi Luan, Ying-Tai Chen

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Peng-Hui Niu, Lu-Lu Zhao, Wan-Qing Wang, Xiao-Jie Zhang, Ze-Feng Li, Xiao-Yi Luan, Ying-Tai Chen, Department of Pancreatic and Gastric Surgery, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China

Corresponding author: Ying-Tai Chen, MD, Department of Pancreatic and Gastric Surgery, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, No. 17 Panjiayuan Nanli, Beijing 100021, China. yingtaichen@126.com

Abstract

BACKGROUND

The impact of racial and regional disparity on younger patients with gastric cancer (GC) remains unclear.

AIM

To investigate the clinicopathological characteristics, prognostic nomogram, and biological analysis of younger GC patients in China and the United States.

METHODS

From 2000 to 2018, GC patients aged less than 40 years were enrolled from the China National Cancer Center and the Surveillance Epidemiology and End Results database. Biological analysis was performed based on the Gene Expression Omnibus database. Survival analysis was conducted *via* Kaplan-Meier estimates and Cox proportional hazards models.

RESULTS

A total of 6098 younger GC patients were selected from 2000 to 2018, of which 1159 were enrolled in the China National Cancer Center, and 4939 were collected from the Surveillance Epidemiology and End Results database. Compared with the United States group, younger patients in China revealed better survival outcomes ($P < 0.01$). For race/ethnicity, younger Chinese cases also enjoyed a better prognosis than that in White and Black datasets ($P < 0.01$). After stratification by pathological Tumor-Node-Metastasis (pTNM) stage, a survival advantage was observed in China with pathological stage I, III, and IV (all $P < 0.01$), whereas younger GC patients with stage II showed no difference ($P = 0.16$). In multivariate analysis, predictors in China involved period of diagnosis, linitis plastica, and pTNM stage, while race, diagnostic period, sex, location, differen-

tiation, linitis plastica, signet ring cell, pTNM stage, surgery, and chemotherapy were confirmed in the United States group. Prognostic nomograms for younger patients were established, with the area under the curve of 0.786 in the China group and of 0.842 in the United States group. Moreover, three gene expression profiles (GSE27342, GSE51105, and GSE38749) were enrolled in further biological analysis, and distinctive molecular characteristics were identified in younger GC patients among different regions.

CONCLUSION

Except for younger cases with pTNM stage II, a survival advantage was observed in the China group with pathological stage I, III, and IV compared to the United States group, which might be partly due to differences in surgical approaches and the improvement of the cancer screening in China. The nomogram model provided an insightful and applicable tool to evaluate the prognosis of younger patients in China and the United States. Furthermore, biological analysis of younger patients was performed among different regions, which might partly explain the histopathological behavior and survival disparity in the subpopulations.

Key Words: Gastric cancer; Younger patients; Racial disparity; Regional disparity; Prediction model; Biological analysis

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Core Tip: The impact of racial and regional disparity on younger patients with gastric cancer (GC) is not clear. A total of 6098 younger GC patients were selected from 2000 to 2018, of which 1159 were enrolled in the China National Cancer Center, and 4939 were collected from the Surveillance Epidemiology and End Results database. Compared with the United States group, younger patients in China revealed better survival outcomes and a better prognosis. Three gene expression profiles from the Gene Expression Omnibus database were enrolled in further biological analysis, and distinctive molecular characteristics were identified in younger GC patients among different regions.

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INTRODUCTION

Gastric cancer (GC) is the fourth leading cause of cancer-related deaths worldwide[1], with the highest incidence among individuals aged 50-70 years. Only 2.0%-6.2% of documented cases are among patients younger than 40 years[2,3]. Although the incidence of GC has gradually declined, important age-specific details may be obscured by the overall trend[4]. During the last decades, the incidence of younger GC patients has remained stable or even increased in both Western and Eastern populations[5-7].

It was long thought that younger patients with GC had aggressive behavior and serious prognosis[8]. Further insight into which subpopulations were at the highest risk of dying remained a crucial requisite so that interventions could be initiated appropriately. Recently, several studies have demonstrated a wide survival discrepancy of GC in different regions or races[9,10]. Notably, GC patients in Asia had more favorable prognoses than patients in Western countries. The important difference has also been found in certain subtypes of GC, including sex and anatomic location[9,10]. For younger GC patients, however, knowledge concerning the regional and racial disparity is scarce. Chen *et al*[11] reported that younger patients in China have a longer survival time than younger patients in the United States, whereas Strong *et al*[12] showed no difference between the United States and Chinese cohorts. The inconsistent findings from these studies might derive from the small sample sizes, with the population records ranging from 336 to 1075.

As such, based on a unique combination of the Surveillance Epidemiology and End Results (SEER) database in the United States and a high-volume National Cancer Center Database in China, we sought to compare the clinicopathological features, survival outcomes, and prognostic nomograms in younger Chinese and United States patients. Moreover, biological analysis of younger GC patients was further evaluated, which might partly explain the histopathological behaviors and survival disparity among different regions and races.

MATERIALS AND METHODS

Data source and patient selection

The study queried clinical data from 2000 to 2018 based on the two large independent cohorts. The histologically confirmed GC cases in China were selected through the China National Cancer Center Gastric Cancer Database (NCCGCDB). As a single but high-volume cohort, NCCGCDB included more than 18000 patients from all regions of China over the past 20 years. Meanwhile, the United States group was identified from the SEER database. The SEER database, supported by the National Cancer Institute, constituted approximately 27.8% of the United States population[13]. Younger patients were defined as GC cases younger than 40 years of age, which remains consistent with the majority of previous studies [14,15]. Clinical data abstracted from the NCCGCDB and SEER databases included younger patients' demographics, clinicopathological characteristics, and survival variables. The stage of GC was assessed according to the 8th edition of the American Joint Committee on Cancer Tumor-Node-Metastasis (TNM) staging system. The primary endpoint of the study was overall survival (OS). Moreover, the gene expression sets evaluated in the study were obtained from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). The study protocol was approved by the ethics committee of National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College (No. 17-156/1412), and all methods were carried out in accordance with relevant guidelines and regulations in the ethics approval and consent.

Statistical analysis

The line chart was plotted to analyze the changing ratios of younger GC patients from 2000 to 2018. Categorical variables were compared using the χ^2 test. Comparisons were performed using the *t*-test for normally distributed continuous variables and the Mann-Whitney *U* test for variables not normally distributed. Survival curves for different regions and races were calculated with the Kaplan-Meier method, while the log-rank test estimated the relevant survival disparity. Univariate and multivariate Cox proportional hazards models were used to determine the prognostic factors for younger patients, while the corresponding hazard ratio and 95% confidence interval (CI) were generated. The covariates with a *P* value of < 0.10 in the univariate models were included in the multivariate analysis[16]. Statistical significance was set at a two-sided *P* value less than 0.05. The survival nomogram for China and the United States was formulated based on the multivariate analysis. Younger cases were randomized 7:3, which were adopted in the training set and the validation set, respectively. The area under the curves (AUC), the concordance index (C-index), and the calibration plots were performed to measure the effectiveness of nomograms. All statistical analyses in the study were conducted using R software v.3.6.3 (http://www.r-project.org/).

Bioinformatics analysis

Based on the GEO database, the differentially expressed genes (DEGs) analysis was further performed to evaluate gene discrepancy for younger patients among different regions, while genes that met the cutoff criteria, adjusted *P*-value < 0.05 and |logFC| > 2.0, where FC represents fold change, were considered as DEGs. A heatmap was constructed with 50 differential genes from the DEGs analysis. The Search Tool for the Retrieval of Interacting Genes database (http://string-db.org/) was designed to analyze the protein-protein interaction (PPI) network, which was subsequently visualized by Cytoscape software (www.cytoscape.org/). As a plugin in Cytoscape, CytoHubba was used to calculate the degree of protein node, and the top 10 genes were identified as hub genes.

RESULTS

Demographic and clinicopathological features

From 2000 to 2018, a total of 6098 younger GC patients were selected, of which 1159 were enrolled in NCCGCDB, and 4939 were collected from the SEER database. The mean age of younger patients was 33.77 ± 4.53 in China, and 32.91 ± 5.38 in the United States ($P < 0.0001$). As shown in Figure 1, time trends of younger patients from 2000 to 2018 have remained stable in both China and the United States sets (range 3.9%-6.7%, range 3.4%-3.8%, respectively). Compared to the United States group, the China group had a higher ratio of younger patients over periods.

Notably, compared to the United States, younger GC patients in China predominantly had distal tumor location (83.7% *vs* 66.7%, $P < 0.0001$), poor differentiation (82.5% *vs* 78.5%, $P < 0.0001$), and signet ring cell carcinoma (52.9% *vs* 28.9%, $P < 0.0001$). Conversely, relatively higher percentages of proximal location (33.3% *vs* 16.3%, $P < 0.0001$), well differentiation (7.5% *vs* 1.2%, $P < 0.0001$), and pTNM stage IV tumors (52.5% *vs* 21.4%, $P < 0.0001$) were revealed in the United States patients (Table 1). As for treatment, the percentage of surgery (75.1% *vs* 41.4%, $P < 0.0001$), lymphadenectomy (67.4% *vs* 19.1%, $P < 0.0001$), and lymphadenectomy with at least 15 examined lymph nodes (ELNs ≥ 15) (52.4% *vs* 10.6%, *P*

Table 1 Demographic and clinicopathological characteristics of younger gastric cancer patients in China and the United States from 2000 to 2018

	China (*n* = 1159), *n* (%)		United States (*n* = 4939), *n* (%)		*P* value
Period of diagnosis					< 0.0001
1 (2000-2003)	194	16.7	972	19.7	
2 (2004-2008)	237	20.5	1248	25.3	
3 (2009-2013)	396	34.3	1280	25.9	
4 (2014-2018)	332	28.6	1439	29.1	
Race					< 0.0001
Chinese	1159	100.0	126	2.6	
White			3405	69.9	
Black			703	14.4	
Others			640	13.1	
Gender					0.135
Male	581	50.1	2599	52.6	
Female	578	49.9	2340	47.4	
Age (year), mean ± SD					0.005
	34		32.91		
	5		5.38		
Primary tumor location					< 0.0001
Proximal	176	16.3	1030	33.3	
Distal	903	83.7	2067	66.7	
Differentiation					< 0.0001
Well	9	1.2	211	7.5	
Moderate	124	16.3	394	14.0	
Poor/Undifferentiated	626	82.5	2214	78.5	
Linitis plastica					0.070
Yes	29	2.6	57	1.7	
No	1101	97.4	3279	98.3	
Signet ring cell carcinoma					< 0.0001
Yes	426	52.9	1429	28.9	
No	380	47.1	3510	71.1	
Pathologic T-stage (AJCC 8th)					< 0.0001
T1	197	22.2	498	24.3	
T2	97	10.9	266	13.0	
T3	143	16.1	509	24.9	
T4	452	50.8	773	37.8	
Pathologic N-stage (AJCC 8th)					< 0.0001
N0	412	41.8	1137	49.9	
N1	119	12.1	724	31.7	
N2	162	16.4	232	10.2	
N3	292	29.6	188	8.2	
Pathologic M-stage					< 0.0001

M0	869	79.4	1268	45.9	
M1	225	20.6	1492	54.1	
pTNM stage (AJCC TNM 8th)					< 0.0001
I	222	21.2	649	20.3	
II	169	16.1	377	11.7	
III	433	41.3	498	15.5	
IV	225	21.4	1687	52.5	
Surgery					< 0.0001
Yes	869	75.1	2047	41.4	
No	288	24.9	2892	58.6	
Chemotherapy					< 0.0001
Yes	428	36.9	2991	60.6	
No	731	63.1	1948	39.4	
Lymphadenectomy					< 0.0001
Yes	780	67.4	941	19.1	
No	377	32.6	3398	80.9	
Lymphadenectomy with at least 15 lymph nodes					< 0.0001
Yes	606	52.4	524	10.6	
No	551	47.6	4415	89.4	
Number nodes examined (*n*), mean ± SD					< 0.0001
	25		18.9		
	13		15.0		
Number positive nodes (*n*), mean ± SD					< 0.0001
	6		7.4		
	8		7.4		

AJCC: American Joint Committee on Cancer; pTNM: Pathological Tumor-Node-Metastasis; SD: Standard deviation.

< 0.0001) were higher in China than those in the United States. In addition, nearly 60.6% of the patients in the United States cohort received chemotherapy compared with only 36.9% in the Chinese cohort (P < 0.0001).

Survival analysis in different regions and races

Survival trends of younger GC patients are summarized in Table 2 and Figure 2. Compared to the stable prognosis of the United States, a noticeable survival increment was shown in younger Chinese patients. The 3-year OS increased from 54.5% (95%CI: 41.7%-71.4%) in 2000-2003 to 66.5% (95%CI: 60.8%-72.7%) in 2014-2018, while the 5-year OS improved from 47.7% (95%CI: 35.0%-65.0%) in 2000-2003 to 51.6% (95%CI: 46.6%-57.2%) in 2009-2013.

Figure 3 depicts the Kaplan-Meier curves for different regions and races. The survival outcomes were much better in Chinese patients or patients diagnosed in China, compared with other races (mentioned in SEER dataset) or the United States patients (all P < 0.0001). To avoid the bias of pathological stage, further analysis was performed to evaluate prognosis of younger cases divided by pTNM stage. The left column of Figure 3 shows the survival curves of younger patients in China and the United States diagnosed as all pTNM stages (Figure 3A), pTNM stage I (Figure 3B), pTNM stage II (Figure 3C), pTNM stage III (Figure 3D), and pTNM stage IV (Figure 3E), while the right column evaluated the OS among different races/ethnicities. Except for stage II GC patients (P = 0.16, P = 0.22, respectively), all other Kaplan-Meier curves revealed obvious survival advantages in patients who were Chinese or diagnosed in China (all P < 0.01).

Moreover, the univariate and multivariate analyses were performed to investigate the prognostic factors for younger GC patients in the China group and the United States group (Tables 3 and 4).

Table 2 Overall survival rates by background characteristics from 2000 to 2018

Characteristics	China			United States		
	1-yr, % (95%CI)	3-yr, % (95%CI)	5-yr, % (95%CI)	1-yr, % (95%CI)	3-yr, % (95%CI)	5-yr, % (95%CI)
Total						
Period of diagnosis						
1 (2000-2003)	75.0 (63.2-89.0)	54.5 (41.7-71.4)	47.7 (35.0-65.0)	58.4 (55.3-61.7)	40.7 (37.7-44.0)	36.2 (33.2-39.4)
2 (2004-2008)	80.1 (74.5-86.1)	59.7 (52.9-67.3)	54.1 (47.4-61.9)	58.1 (55.4-61.0)	42.3 (39.6-45.2)	38.4 (35.7-41.3)
3 (2009-2013)	76.7 (72.3-81.3)	57.1 (52.1-62.6)	51.6 (46.6-57.2)	60.8 (58.1-63.6)	44.0 (41.2-46.9)	39.7 (37.0-42.6)
4 (2014-2018)	88.6 (85.1-92.3)	66.5 (60.8-72.7)		61.6(58.9-64.4)	43.0 (39.9-46.4)	
Race						
Chinese	81.4 (78.9-84.1)	60.9 (57.6-64.3)	52.3 (48.8-56.0)	66.0 (58.1-75.1)	52.9 (44.4-62.9)	42.9 (34.4-53.5)
White				59.7 (58.1-61.5)	41.7 (40.0-43.6)	37.8 (36.0-39.6)
Black				58.0 (54.3-61.8)	42.7 (39.0-46.7)	38.2 (34.6-42.3)
Others				59.4 (55.6-63.5)	43.3 (39.4-47.6)	37.2 (33.3-41.6)
Gender						
Male	82.2 (78.7-85.8)	61.5 (57.1-66.2)	53.6 (49.0-58.7)	57.0 (55.0-59.0)	39.6 (37.6-41.7)	34.8 (32.8-36.9)
Female	80.6 (76.9-84.5)	60.2 (55.5-65.3)	50.7 (45.7-56.2)	63.0 (61.0-65.1)	46.2 (44.1-48.4)	42.2 (40.1-44.4)
Primary tumor location						
Proximal	76.7 (69.8-84.2)	47.7 (39.8-57.2)	42.7 (34.7-52.4)	59.0 (56.0-62.2)	35.8 (32.8-39.1)	30.5 (27.5-33.7)
Distal	84.5 (81.8-87.3)	66.3 (62.8-70.1)	57.4 (53.5-61.5)	64.6 (62.5-66.7)	48.0 (45.8-50.4)	42.9 (40.6-45.3)
Differentiation						
Well	100.0 (100.0-100.0)	100.0 (100.0-100.0)	85.7 (63.3-100.0)	92.5 (88.9-96.2)	83.3 (78.0-89.0)	78.8 (72.6-85.6)
Moderate	90.4 (84.7-96.6)	76.1 (67.6-85.6)	66.3 (56.4-78.0)	72.3 (67.9-76.9)	50.1 (45.2-55.5)	44.6 (39.6-50.1)
Poor/Undifferentiated	87.7 (84.8-90.7)	69.6 (65.4-74.0)	61.6 (57.0-66.5)	48.3 (46.3-50.5)	25.7 (23.9-27.6)	19.9 (18.2-21.8)
Linitis plastica						
Yes	57.1 (39.5-82.8)	14.3 (5.0-40.7)	4.8 (0.7-32.2)	49.5 (37.8-65.0)	14.8 (7.6-29.0)	12.3 (5.8-26.4)
No	83.9 (81.4-86.6)	68.7 (65.4-72.2)	63.8 (60.2-67.5)	60.0 (58.6-61.4)	43.1 (41.6-44.6)	38.6 (37.1-40.1)
Signet ring cell carcinoma						
Yes	91.0 (87.9-94.2)	80.3 (75.8-85.0)	76.9 (72.1-82.1)	49.5 (37.8-65.0)	14.8 (7.6-29.0)	12.3 (5.8-26.4)
No	90.2 (86.7-93.8)	75.1 (70.0-80.6)	70.8 (65.2-76.8)	60.0 (58.6-61.4)	43.1 (41.6-44.6)	38.6 (37.1-40.1)
pTNM stage (AJCC TNM 8th)						
I	100.0 (100.0-100.0)	99.4 (98.4-100.0)	99.4 (98.4-100.0)	91.5 (89.3-93.8)	86.4 (83.6-89.2)	83.4 (80.2-86.6)
II	97.1 (94.3-99.9)	83.6 (77.5-90.3)	69.8 (61.9-78.7)	84.2 (80.4-88.2)	68.4 (63.4-73.8)	61.0 (55.5-67.0)
III	83.5 (79.4-87.9)	54.9 (49.3-61.0)	42.6 (37.0-49.2)	74.8 (70.9-78.8)	41.0 (36.5-46.1)	29.6 (25.3-34.7)
IV	51.2 (43.8-59.7)	17.4 (12.3-24.8)	9.9 (6.0-16.3)	29.7 (27.5-32.1)	10.4 (8.8-12.1)	7.1 (5.7-8.7)
Surgery						
Yes	89.6 (87.3-92.0)	72.4 (69.0-76.0)	63.4 (59.5-67.5)	82.6 (80.9-84.3)	61.2 (59.0-63.5)	53.6 (51.3-56.1)
No	58.4 (51.8-65.9)	33.4 (26.9-41.5)	28.7 (22.2-37.0)	43.2 (41.3-45.1)	29.1 (27.4-31.0)	27.0 (25.3-28.9)
Chemotherapy						
Yes	89.1 (85.8-92.6)	67.0 (61.9-72.6)	52.7 (47.1-59.1)	55.9 (54.1-57.8)	32.7 (31.0-34.6)	27.4 (25.7-29.2)
No	76.8 (73.3-80.4)	57.2 (53.1-61.6)	51.9 (47.7-56.5)	66.4 (64.2-68.6)	59.2 (56.9-61.6)	56.2 (53.9-58.7)

Lymphadenectomy						
Yes	90.8 (88.4-93.2)	77.7 (74.3-81.3)	73.7 (69.9-77.6)	70.9 (67.9-73.9)	39.4 (36.2-42.8)	29.2 (26.1-32.6)
No	64.0 (58.3-70.3)	41.4 (35.4-48.5)	35.1 (29.0-42.5)	57.2 (55.6-58.8)	43.7 (42.1-45.4)	40.7 (39.1-42.4)
Lymphadenectomy with at least 15 lymph nodes						
Yes	91.8 (89.3-94.3)	79.9 (76.2-83.3)	76.2 (72.2-80.6)	77.3 (73.7-81.1)	43.4 (39.4-48.6)	32.4 (28.1-37.2)
No	86.5 (80.4-93.1)	69.0 (60.9-78.3)	64.1 (55.6-73.8)	63.0 (58.4-67.9)	34.0 (29.6-39.1)	25.3 (21.2-30.2)

AJCC: American Joint Committee on Cancer; CI: Confidence interval pTNM: Pathological Tumor-Node-Metastasis.

Significant variables on univariate analysis were enrolled in the multivariate modeling, followed by region, race, period of diagnosis, sex, location, differentiation, linitis plastica, signet ring cell carcinoma, pTNM, surgery, chemotherapy, ELNs ≥ 15 (all $P < 0.05$). For younger GC patients, the independent prognosis factors included region, period of diagnosis, poorly differentiated, linitis plastica, pTNM stage, surgery, chemotherapy, and ELNs ≥ 15 (all $P < 0.05$). However, significant predictors in China only involved period of diagnosis (all $P < 0.05$), linitis plastica ($P = 0.0005$), and pTNM stage (all $P < 0.0001$). For the United States patients, Black ethnicity, poorly differentiated, linitis plastica, signet ring cell, and later pathological stage were related to serious prognosis (all $P < 0.01$), whereas recent period, female, distal location, surgery, and chemotherapy emerged as protective factors (all $P < 0.05$).

Nomogram of younger GC patients

To predict OS of younger patients with GC, nomograms for China and the United States group were established separately based on the results of the Cox regression analysis. As illustrated in Figure 4, a total of four clinical parameters (including linitis plastica, surgery, ELNs ≥ 15, and pTNM stage) were included in the Chinese models, while the nomogram for the United States identified the following parameters: Race, sex, tumor location, differentiation, pTNM stage, linitis plastica, signet ring cell, surgery, and chemotherapy.

According to the results of the validation set, the C-index for the China and United States models was 0.814 and 0.787, respectively. The AUC that was applied to evaluate the discernment of the nomogram models was 0.786 in the China group and 0.842 in the United States group (Supplementary Figure 1). In addition, high-quality calibration plots in both China and the United States models had been demonstrated (Supplementary Figure 2).

DEGs analysis, PPI network, and hub gene identification from GEO database

To evaluate gene disparities according to different races, we performed DEGs analysis for younger GC patients from the GEO database. Three gene expression profiles (GSE27342, GSE51105, and GSE38749), which contained 8 younger cases from China, and 12 samples from the Americas and Oceania, were included in this study. After DEGs analysis was performed among different regional sets, 50 significant DEGs were selected (Figure 5). Compared to other regions, certain genes were downregulated in China (Figure 5A), while *C11orf58, TLK1, ARHGAP5, MRPL2,* and *RBMX2* were upregulated. Moreover, the PPIs among the DEGs were performed with the Search Tool for the Retrieval of Interacting Genes. A total of 200 nodes and 245 edges were involved in the PPI network (Figure 5B and C). The top 10 genes evaluated by connectivity degree in the PPI network were as follows: *CTNNB1, APP, CTSB, UBA52, RHOA, ATP5B, EEF2, FN1, RPL11,* and *CTSD*.

DISCUSSION

Our study compared younger GC patients in China and the United States at clinical and biological levels. Survival advantages of younger GC patients in China were demonstrated from two high-volume databases. These data were stronger than previous studies[17], and high-quality nomograms were further constructed for younger patients according to different regions. Compared to western regions, DEGs in China were confirmed, which might partly explain the histopathological behaviors and survival disparity.

Staying in line with the previous results[11,18], our conclusions showed that younger patients in China were more prominent in the distal location, while GC in the proximal location was more common in the United States. The above discrepancy was presumably related to certain predisposing factors[19-21]. In Western countries, obesity and gastroesophageal reflux were associated with proximal GC[20], whereas *Helicobacter pylori* infection in China may partially account for a high incidence of the distal location[21]. Additionally, Chinese cases were more likely diagnosed with an early pathological stage than those in the United States. This might partially be due to the improvement of cancer screening and

Table 3 Univariate Cox regression analysis of younger gastric cancer patients in China and the United States

Characteristics		Total		*P* value	China		*P* value	United States		*P* value	China *vs* United States		*P* value
		HR	95%CI		HR	95%CI		HR	95%CI		HR	95%CI	
Region	China	1											
	United States	2.06	1.84-2.31	< 0.0001									
Race	Chinese	1						1			1.24	1.00-1.54	0.11
	White	2.08	1.85-2.33	< 0.0001				1.22	0.95-1.56	0.12			
	Black	2.14	1.85-2.47	< 0.0001				1.26	0.97-1.63	0.08			
	Others	2.07	1.79-2.38	< 0.0001				1.26	0.97-1.64	0.08			
Period of diagnosis	Period 1 (2000-2003)	1			1			1			1.12	0.83-1.49	0.55
	Period 2 (2004-2008)	0.90	0.81-0.99	0.009	0.67	0.45-1.00	0.05	0.95	0.85-1.05	0.30	1.52	1.27-1.80	< 0.0001
	Period 3 (2009-2013)	0.87	0.78-0.96	0.008	0.84	0.57-1.22	0.35	0.91	0.82-1.01	0.09	1.36	1.19-1.55	< 0.0001
	Period 4 (2014-2018)	0.84	0.76-0.94	0.008	0.74	0.49-1.10	0.14	0.91	0.82-1.02	0.10	2.03	1.72-2.41	< 0.0001
Gender	Male	1			1			1			1.70	1.51-1.91	< 0.0001
	Female	0.85	0.80-0.91	< 0.0001	1.05	0.87-1.26	0.64	0.82	0.76-0.88	< 0.0001	1.52	1.27-1.80	< 0.0001
Primary tumor location	Proximal	1			1			1			1.30	1.08-1.57	0.02
	Distal	0.68	0.62-0.75	< 0.0001	0.59	0.47-0.75	< 0.0001	0.77	0.70-0.85	< 0.0001	1.56	1.40-1.74	< 0.0001
Differentiation	Well	1			1			1			1.10	0.33-3.62	0.90
	Moderate	2.82	2.05-3.88	< 0.0001	1.62	0.39-6.76	0.51	3.17	2.28-4.42	< 0.0001	2.14	1.56-2.94	< 0.0001
	Poor/Undifferentiated	5.27	3.91-7.11	< 0.0001	2.19	0.54-8.83	0.27	6.15	4.53-8.35	< 0.0001	3.20	2.83-3.62	< 0.0001
Linitis plastica	Yes	1.95	1.58-2.40	< 0.0001	3.69	2.41-5.65	< 0.0001	1.89	1.41-2.52	< 0.0001	1.03	0.66-1.60	0.91
	No	1			1			1			2.25	2.03-2.50	< 0.0001
Signet ring cell carcinoma	Yes	1.61	1.52-1.72	< 0.0001	0.80	0.61-1.05	0.18	2.01	1.86-2.17	< 0.0001	6.53	5.33-7.99	< 0.0001
	No	1			1			1			2.45	2.02-2.96	< 0.0001
pTNM stage (AJCC TNM 8th)	1			1	1			1			9.88	4.28-22.83	< 0.0001
	2.85	2.25-3.60	< 0.0001	2.85	19.50	7.04-54.05	< 0.0001	2.27	1.76-2.93	< 0.0001	1.27	0.96-1.67	0.16
	6.18	5.04-7.58	< 0.0001	6.18	46.77	17.36-125.96	< 0.0001	4.88	3.93-6.07	< 0.0001	1.31	1.13-1.53	0.003
	19.52	16.09-23.68	< 0.0001	19.52	137.73	50.88-372.83	< 0.0001	14.25	11.69-17.36	< 0.0001	1.46	1.26-1.68	< 0.0001

Surgery	Yes	0.36	0.34-0.38	< 0.0001	0.23	0.19-0.28	< 0.0001	0.41	0.37-0.44	< 0.0001	1.29	1.15-1.45	0.0002
	No	1			1			1			1.25	1.08-1.25	0.01
Chemotherapy	Yes	1.71	1.59-1.84	< 0.0001	0.93	0.77-1.13	0.45	1.80	1.66-1.95	< 0.0001	2.10	1.84-2.40	< 0.0001
	No	1			1			1			0.97	0.87-1.09	0.69
Lymphadenectomy with at least 15 lymph nodes	Yes	0.56	0.49-0.63	< 0.0001	0.64	0.47-0.87	< 0.0001	0.73	0.63-0.86	0.0001	2.49	2.12-2.91	< 0.0001
	No	1			1			1			3.86	3.21-4.65	< 0.0001

AJCC: American Joint Committee on Cancer; CI: Confidence interval; HR: Hazard ratio; pTNM: Pathological Tumor-Node-Metastasis.

early detection programs in China, which have expanded to 31 provinces since 2015[22,23]. When considering surgical patterns, it was thought that gastrectomy with D2 lymphadenectomy was the standard treatment for GC in Eastern Asia[24]. However, most patients in Western countries undergo D1 lymphadenectomy, and D2 lymphadenectomy was only recommended rather than the therapeutic norm[25,26]. These treatment differences might explain the higher percentage of ELNs ≥ 15 and the larger number of nodes examined for Chinese younger patients in our study.

With a direct comparison from a high-volume GC cohort in China and the SEER database, we reported significant survival differences for younger GC patients. Consistent with the previous studies [11,18], our findings revealed that younger cases had a better prognosis in China than in the United States. With the exception of pTNM stage II, significantly better OS was observed in the China group with stages I, III, and IV. Notably, the survival advantage of younger Chinese patients could partially be attributed to a remarkable improvement in the quality of clinical services, such as improved access to primary healthcare, early cancer screening, and individual multimodal therapies[22,23,27]. Moreover, it was well known that gastrectomy with D2 lymphadenectomy was common in Asian areas, while the vast majority of GC cases in Western countries undergo D1 lymphadenectomy[24]. The above type of surgical procedures might be associated with the survival disparity among different regions. In addition, the later tumor stage and some other factors including lifestyle and high body mass index in the United States patients might also in part explain the poor prognosis[28].

As a convenient statistical predictive tool, nomograms have been widely applied for physicians to clarify a diagnosis and predict survival[29,30]. To our best knowledge, the present study, which used the largest sample sizes, constructed survival nomograms for younger GC patients in China and the United States. Compared with previous models[18], nomograms not only avoid the bias of regions but also achieve reliable predictive performance, which was reflected by an AUC of 0.786 for China and 0.842 for the United States. In addition, the calibration plots of the training set and validation set (Supplementary Figure 1) illustrated great agreement between nomogram prediction and actual observation, thus further suggesting a robust predictive ability of nomograms in the present study.

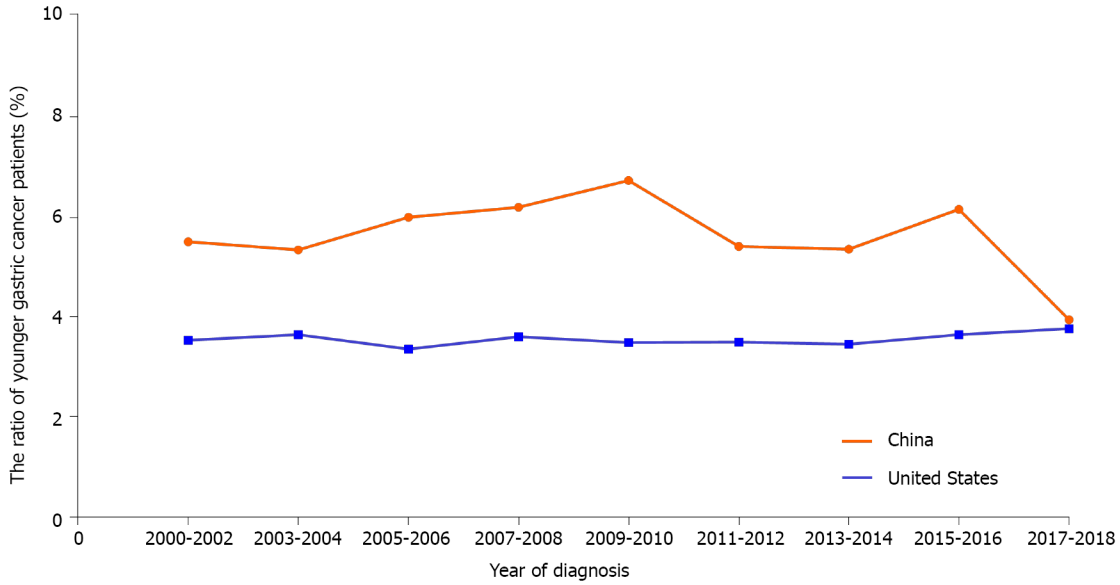
Although specific gene expression among regions and races was not yet fully ascertained, several studies demonstrated a strong association between regional/racial-related genes and the prognosis of GC[31,32]. Loh *et al*[31] reviewed the genetic polymorphisms of GC among different races/ethnicities. The results showed that 37 polymorphisms across 27 genes were significantly related to GC in Asians,

Table 4 Multivariate Cox regression analysis of younger gastric cancer patients in China and the United States

Characteristics		Total[1]		*P* value	China[2]		*P* value	United States[3]		*P* value
		HR	95%CI		HR	95%CI		HR	95%CI	
Region	China	1								
	United States	2.42	1.35-4.33	0.01						
Race	Chinese	1						1		
	White	0.80	0.45-1.41	0.52				1.21	0.94-1.55	0.13
	Black	0.84	0.46-1.53	0.63				1.48	1.14-1.92	0.003
	Others	0.76	0.42-1.38	0.45				1.19	0.91-1.55	0.20
Period of diagnosis	Period 1 (2000-2003)	1			1			1		
	Period 2 (2004-2008)	0.62	0.27-0.78	0.02	0.51	0.29-0.89	0.047	0.55	0.47-0.64	< 0.0001
	Period 3 (2009-2013)	0.54	0.25-0.73	0.01	0.39	0.22-0.67	0.004	0.50	0.43-0.59	< 0.0001
	Period 4 (2014-2018)	0.48	0.16-0.49	0.0002	0.07	0.03-0.14	< 0.0001	0.47	0.40-0.56	< 0.0001
Gender	Male	1						1		
	Female	0.92	0.80-1.07	0.35				0.85	0.79-0.92	< 0.0001
Primary tumor location	Proximal	1			1			1		
	Distal	0.92	0.75-1.12	0.47	0.88	0.60-1.29	0.58	0.88	0.80-0.98	0.02
Differentiation	Well	1						1		
	Moderate	2.89	0.88-9.48	0.14				1.90	1.36-2.65	0.0002
	Poor/Undifferentiated	4.77	1.47-15.44	0.03				2.97	2.16-4.07	< 0.0001
Linitis plastica	Yes	1.73	1.12-2.68	0.04	6.70	2.72-16.50	0.0005	1.63	1.21-2.19	0.001
	No/unknown	1			1			1		
Signet ring cell carcinoma	Yes	1.07	0.92-1.24	0.47				1.48	1.36-1.61	< 0.0001
	No/unknown	1						1		
pTNM stage (AJCC TNM 8th)	I	1			1			1		
	II	10.96	5.05-23.75	< 0.0001	18.69	5.55-62.96	< 0.0001	2.35	1.81-3.04	< 0.0001
	III	27.32	12.87-58.00	< 0.0001	58.95	18.18-191.13	< 0.0001	4.51	3.57-5.69	< 0.0001
	IV	57.20	26.58-123.10	< 0.0001	158.6	43.85-573.93	< 0.0001	8.26	6.72-10.16	< 0.0001
Surgery	Yes	0.50	0.36-0.70	0.0005	0.46	0.20-1.03	0.11	0.42	0.37-0.46	< 0.0001
	No	1			1			1		
Chemotherapy	Yes	0.69	0.58-0.84	0.001				0.82	0.75-0.90	< 0.0001
	No	1						1		
Lymphadenectomy with at least 15 lymph nodes	Yes	0.74	0.63-0.86	0.001	0.88	0.64-1.20	0.49	1.04	0.90-1.20	0.64
	No	1			1			1		

[1]Adjusted factors for total patients: region, race, period, sex, primary tumor location, differentiation, linitis plastica, signet ring cell carcinoma, pathological Tumor-Node-Metastasis (pTNM) stage, surgery, chemotherapy, lymphadenectomy with at least 15 lymph nodes.
[2]Adjusted factors for patients from China cohort: period, primary tumor location, linitis plastica, pTNM stage, surgery, lymphadenectomy with at least 15 lymph nodes.
[3]Adjusted factors for patients from the United States cohort: race, period, sex, primary tumor location, differentiation, linitis plastica, signet ring cell carcinoma, pTNM stage, surgery, chemotherapy, lymphadenectomy with at least 15 lymph nodes.
AJCC: American Joint Committee on Cancer; CI: Confidence interval; HR: Hazard ratio.

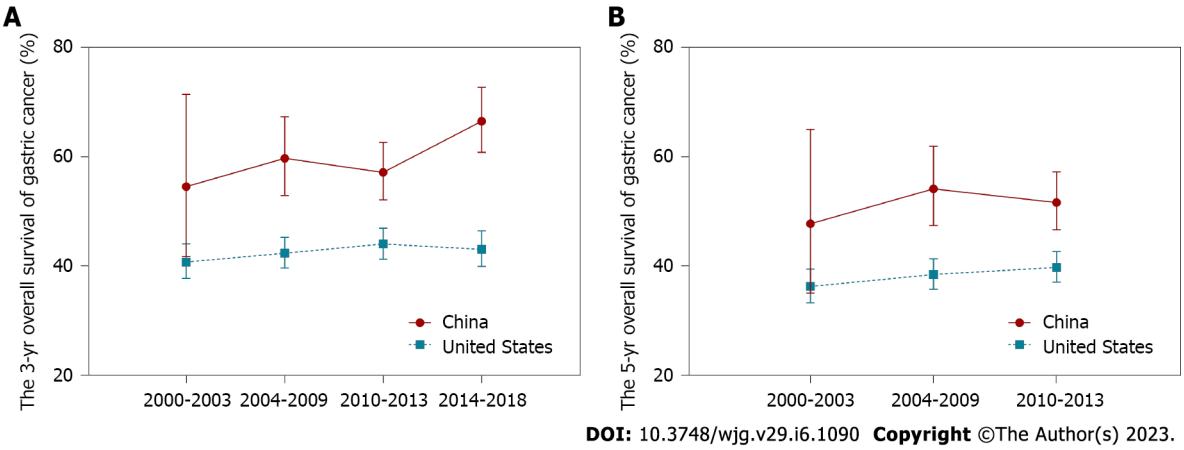
while 12 polymorphisms across 11 genes were found in Caucasians. Then, Li *et al*[32] found significant gene disparity among White, Black, and Asian patients. Four core genes, including *GYG2P1*, *RPS4Y1*, *TXLNG*, and *EIF1AX*, were demonstrated in White ethnicity, which were relevant for RNA binding and



Group	2000-2002	2003-2004	2005-2006	2007-2008	2009-2010	2011-2012	2013-2014	2015-2016	2017-2018
China	5.50%	5.34%	5.99%	6.19%	6.73%	5.41%	5.35%	6.15%	3.94%
United States	3.53%	3.64%	3.35%	3.60%	3.48%	3.49%	3.45%	3.64%	3.76%

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Figure 1 The ratio of younger patients with gastric cancer based on the China National Cancer Center and Surveillance Epidemiology and End Results databases from 2000 to 2018.

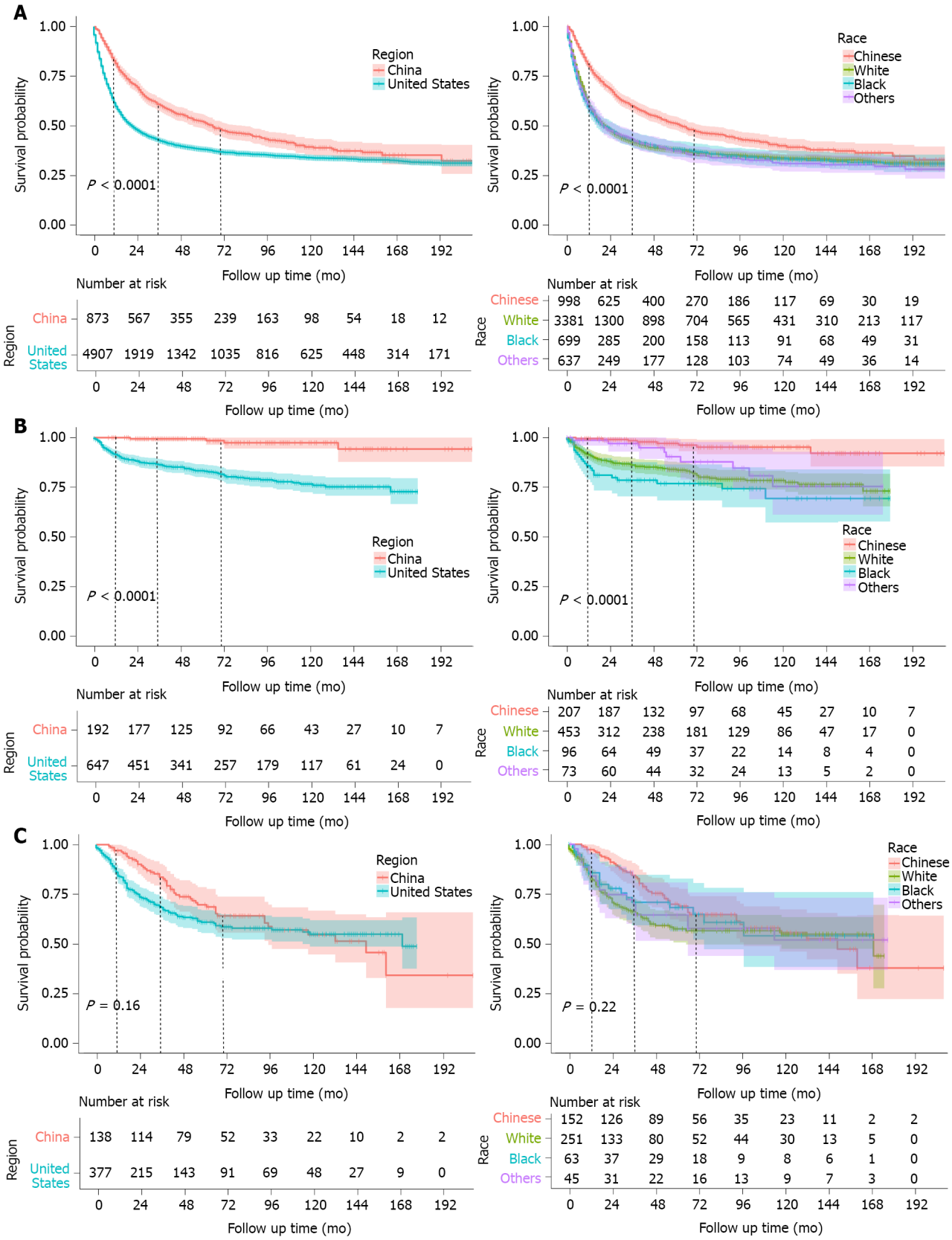


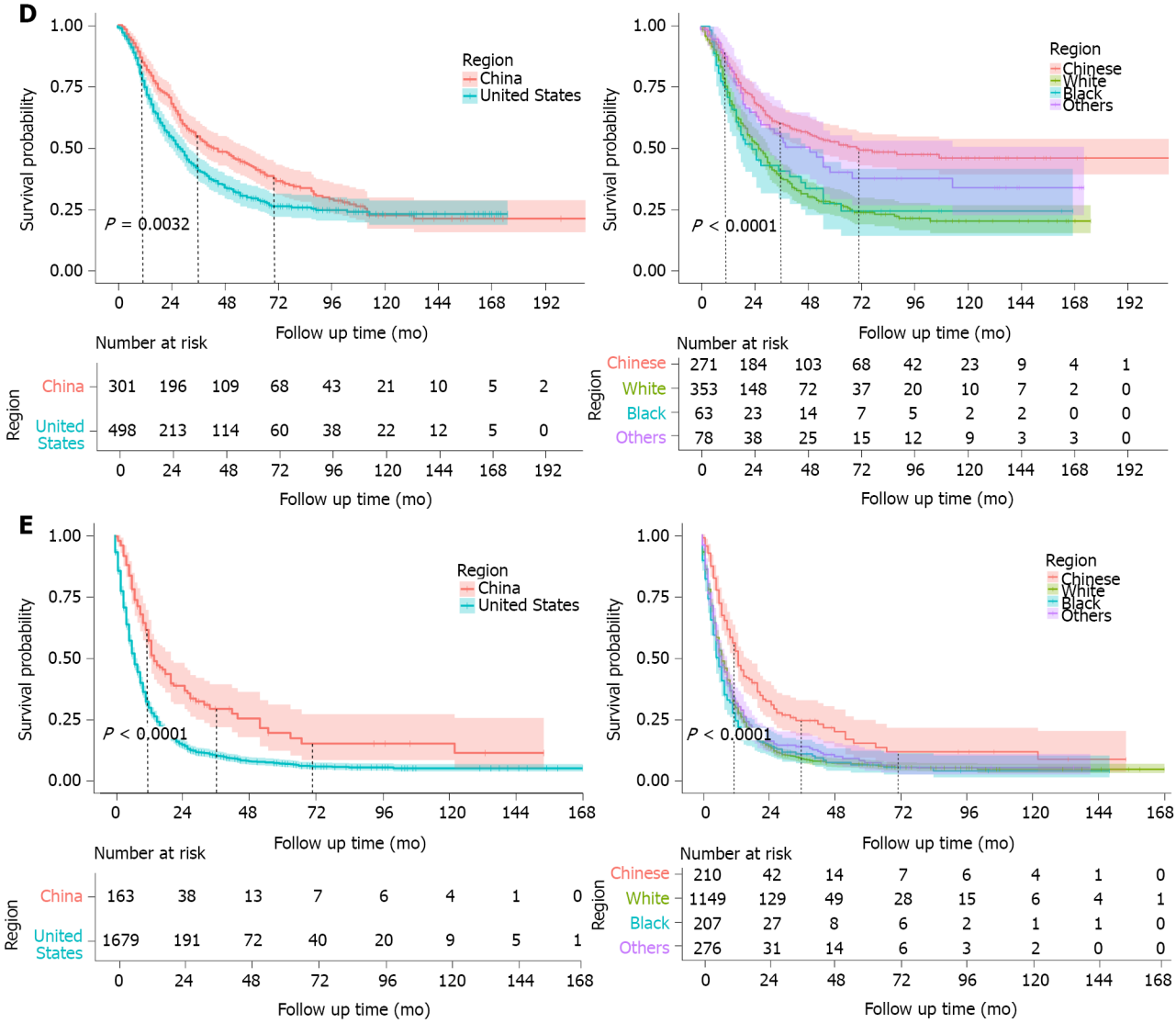
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Figure 2 The survival trends of younger patients with gastric cancer based on the China National Cancer Center and Surveillance Epidemiology and End Results databases. A: Trends of the 3-year overall survival; B: Trends of the 5-year overall survival.

transcription pathways. Black ethnicity with GC was mainly enriched in cell structural changes, and *DNAJC5*, *HDAC10*, *NEO1*, and *SMG5* were identified. For Asian patients, *TMSB4Y*, *UTY*, *ZFY*, and *ZNF787* were screened based on the relationship between gene expression and DNA methylation. In our study, we first evaluated the race/ethnicity-associated genes for younger GC patients. After the construction of PPI networks and screening according to the degree of the protein node, the hub genes of younger GC patients were as follows: *CTNNB1*, *APP*, *CTSB*, *UBA52*, *RHOA*, *ATP5B*, *EEF2*, *FN1*, *RPL11*, and *CTSD*. Further mechanistic studies are warranted to confirm the related signaling and action mechanisms.

Our study has certain strengths. First, two high-volume databases based on the SEER and the NCCGCDB were comprehensively evaluated for the regional and racial disparity of younger GC patients. Second, our study, utilizing the largest sample sizes globally, constructed prognostic nomograms for younger GC patients in China and the United States, thus providing an intuitive accessible tool for physicians to predict survival and develop resurvey schedules. Moreover, to our best knowledge, it was the first study to perform biological analysis for younger patients among different


A
Survival probability
1.00
0.75
0.50
0.25
0.00
Region
China
United States
P < 0.0001
0 24 48 72 96 120 144 168 192
Follow up time (mo)
Number at risk
Region
China 873 567 355 239 163 98 54 18 12
United States 4907 1919 1342 1035 816 625 448 314 171
Race
Chinese
White
Black
Others
P < 0.0001
Chinese 998 625 400 270 186 117 69 30 19
White 3381 1300 898 704 565 431 310 213 117
Black 699 285 200 158 113 91 68 49 31
Others 637 249 177 128 103 74 49 36 14
B
China 192 177 125 92 66 43 27 10 7
United States 647 451 341 257 179 117 61 24 0
Chinese 207 187 132 97 68 45 27 10 7
White 453 312 238 181 129 86 47 17 0
Black 96 64 49 37 22 14 8 4 0
Others 73 60 44 32 24 13 5 2 0
C
P = 0.16
China 138 114 79 52 33 22 10 2 2
United States 377 215 143 91 69 48 27 9 0
P = 0.22
Chinese 152 126 89 56 35 23 11 2 2
White 251 133 80 52 44 30 13 5 0
Black 63 37 29 18 9 8 6 1 0
Others 45 31 22 16 13 9 7 3 0



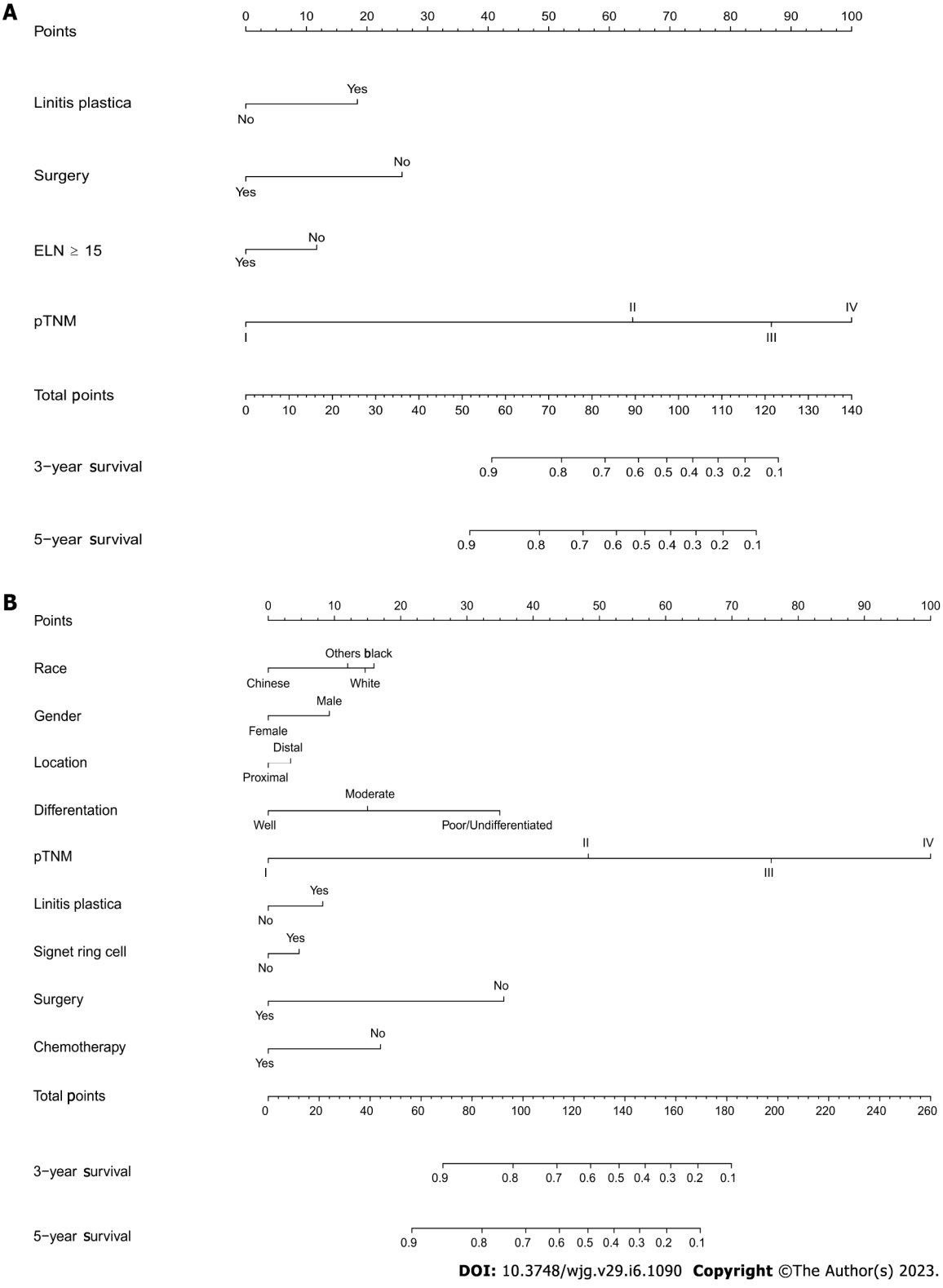
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Figure 3 Kaplan-Meier survival curves of different regions and races from the China National Cancer Center and Surveillance Epidemiology and End Results databases from 2000 to 2018. A: All gastric cancer; B: Gastric cancer diagnosed as Tumor-Node-Metastasis (TNM) stage I; C: Gastric cancer diagnosed as TNM stage II; D: Gastric cancer diagnosed as TNM stage III; E: Gastric cancer diagnosed as TNM stage IV.

regions, which provided a molecular interpretation for the survival discrepancy. Despite all this, several limitations need to be considered in the present study. Some of the key baseline prognostic factors, including body mass index, smoking, drinking, and the efficacy of neo/adjuvant therapy were absent in the SEER database. In addition, as a high-volume single center, some findings from NCCGCDB might not be strongly generalizable to China, which should be replicated in a larger multicenter experience. Lastly, even after correcting by batch effects (Supplementary Figure 3), there was also potential bias during encoding samples from GEO database. These factors might affect the accuracy of the results.

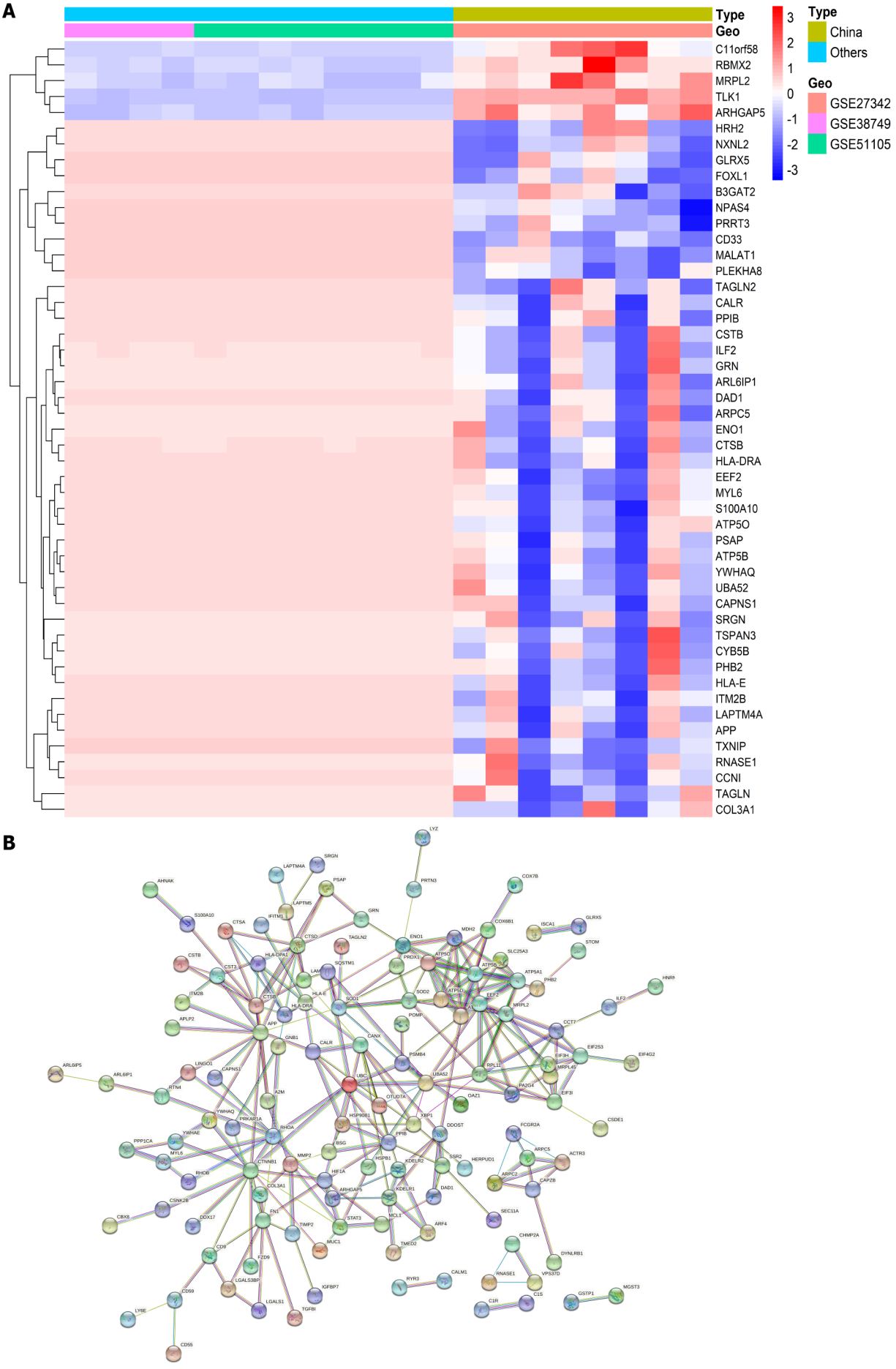
CONCLUSION

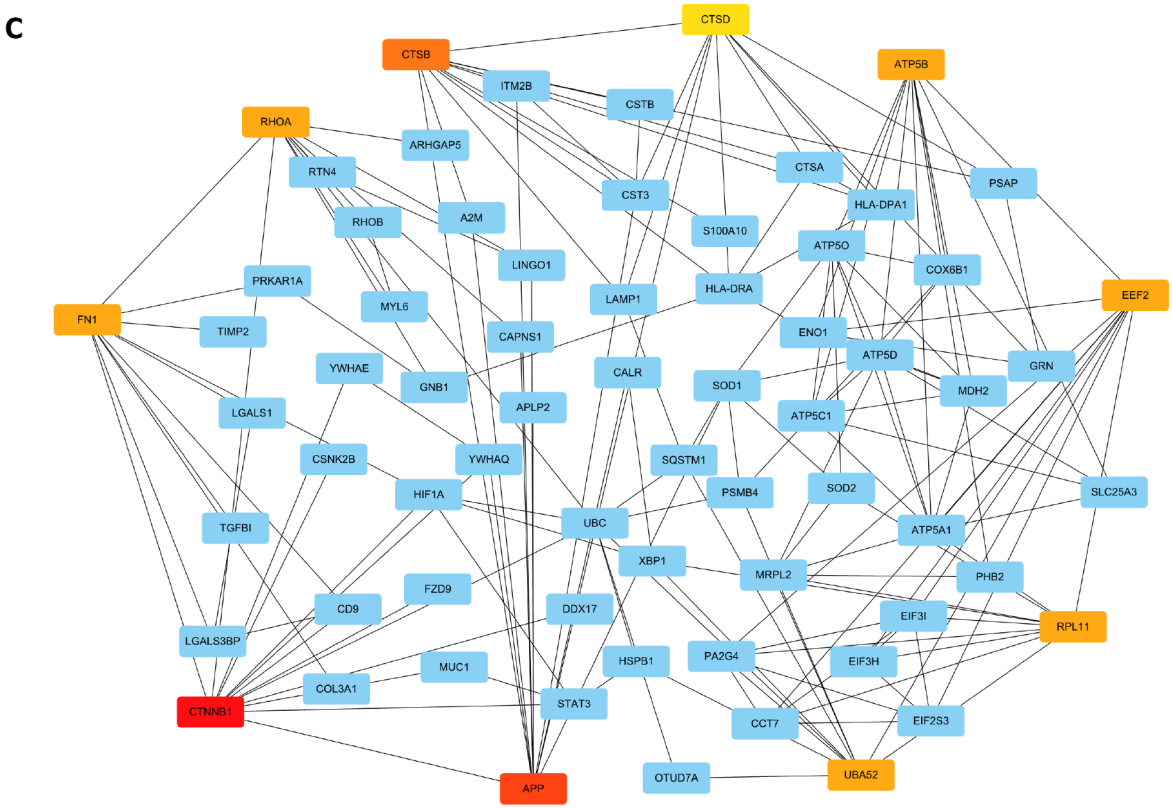
In conclusion, younger GC patients diagnosed in China had a better prognosis than those in the United States. Except for younger cases with pTNM stage II, a survival advantage was observed in the China group with pathological stages I, III, and IV. Moreover, utilizing the largest sample sizes globally, prognostic nomograms in China and the United States were constructed and showed robust predictive performance. Further large-scale studies are warranted to investigate more molecular characteristics and related mechanisms for younger GC patients among different regions and races.



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Figure 4 Prognostic nomogram for younger gastric cancer patients. A: Nomogram based on the China National Cancer Center; B: Nomogram based on the Surveillance Epidemiology and End Results database. ELN: Examined lymph nodes; pTNM: Pathological Tumor-Node-Metastasis.


A
Type
Geo
C11orf58
RBMX2
MRPL2
TLK1
ARHGAP5
HRH2
NXNL2
GLRX5
FOXL1
B3GAT2
NPAS4
PRRT3
CD33
MALAT1
PLEKHA8
TAGLN2
CALR
PPIB
CSTB
ILF2
GRN
ARL6IP1
DAD1
ARPC5
ENO1
CTSB
HLA-DRA
EEF2
MYL6
S100A10
ATP5O
PSAP
ATP5B
YWHAQ
UBA52
CAPNS1
SRGN
TSPAN3
CYB5B
PHB2
HLA-E
ITM2B
LAPTM4A
APP
TXNIP
RNASE1
CCNI
TAGLN
COL3A1
3
2
1
0
-1
-2
-3
Type
China
Others
Geo
GSE27342
GSE38749
GSE51105
B



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Figure 5 After differentially expressed genes analysis was performed among different regional sets, 50 significant differently expressed genes were selected. A: Heatmap constructed with the differentially expressed genes for younger gastric cancer patients; B: Protein–protein interaction network; C: Top 10 hub genes with higher degree of connectivity.

ARTICLE HIGHLIGHTS

Research background

The impact of racial and regional disparity on younger patients with gastric cancer (GC) remains unclear.

Research motivation

This study aimed to provide a national view of younger GC patients in China and the United States.

Research objectives

To investigate the clinicopathological characteristics, prognostic nomogram, and biological analysis of younger GC patients in China and the United States.

Research methods

From 2000 to 2018, GC patients aged less than 40 years were selected from the China National Cancer Center and the Surveillance Epidemiology and End Results database. Biological analysis was enrolled from the Gene Expression Omnibus database.

Research results

A total of 6098 younger GC patients were selected from 2000 to 2018, of which 1159 were enrolled in the China National Cancer Center, and 4939 were collected from the Surveillance Epidemiology and End Results database. Compared with the United States group, younger patients in China revealed better survival outcomes ($P < 0.01$). For race/ethnicity, younger Chinese cases also enjoyed a better prognosis than that in White and Black subsets ($P < 0.01$). Prognostic nomograms for younger patients were established, with area under the curves of 0.786 for China and 0.842 for the United States. Moreover, three gene expression profiles (GSE27342, GSE51105, and GSE38749) were enrolled in further biological analysis, and distinctive molecular characteristics were identified in younger GC patients in different regions.

Research conclusions

Except for younger cases with pathological Tumor-Node-Metastasis stage II, a survival advantage was observed in the China group with pathological stages I, III, and IV. Biological analysis of younger patients was performed among different regions, which might partly explain the histopathological behaviors and survival disparity in the subpopulations.

Research perspectives

Further large-scale studies are warranted to investigate more molecular characteristics and related mechanisms for younger GC patients among different regions and races.

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FOOTNOTES

Author contributions: Niu PH, Zhao LL, and Wang WQ contributed equally to this work and share first authorship. All authors made substantial contributions to the intellectual content of this paper.

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Institutional review board statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the ethics committee of National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College (No. 17-156/1412).

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: All authors report no relevant conflicts of interest for this article.

Data sharing statement: The data used to support the findings of this study are included within the article in Tables.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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Country/Territory of origin: China

ORCID number: Peng-Hui Niu 0000-0003-0114-1625; Lu-Lu Zhao 0000-0001-8344-0498; Wan-Qing Wang 0000-0001-6697-202X; Xiao-Jie Zhang 0000-0001-9850-9806; Ze-Feng Li 0000-0002-5345-3527; Xiao-Yi Luan 0000-0002-1806-8463; Ying-Tai Chen 0000-0003-4980-6315.

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SYSTEMATIC REVIEWS

Relation of COVID-19 with liver diseases and their impact on healthcare systems: The Portuguese case

Sara Fernandes, Milaydis Sosa-Napolskij, Graça Lobo, Isabel Silva

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Sara Fernandes, Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto (ICBAS-UP), Porto 4050-313, Portugal

Milaydis Sosa-Napolskij, CINTESIS@RISE, Center for Health Technology and Services Research at The Associate Laboratory RISE–Health Research Network, Faculty of Medicine of The University of Porto, Porto 4200-219, Portugal

Graça Lobo, Isabel Silva, Laboratory of Pharmacology and Neurobiology–Department of Immuno-physiology and Pharmacology, Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto (ICBAS-UP), Porto 4050-313, Portugal

Graça Lobo, Isabel Silva, Center for Drug Discovery and Innovative Medicines (MedInUP), Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto (ICBAS-UP), Porto 4050-313, Portugal

Corresponding author: Isabel Silva, Doctor, PhD, Researcher, Laboratory of Pharmacology and Neurobiology–Department of Immuno-physiology and Pharmacology, Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto (ICBAS-UP), Rua Jorge Viterbo Ferreira, nº228, Porto 4050-313, Portugal. isabel.silva@ibmc.up.pt

Abstract

BACKGROUND

The impact caused by the coronavirus disease 2019 (COVID-19) on the Portuguese population has been addressed in areas such as clinical manifestations, frequent comorbidities, and alterations in consumption habits. However, comorbidities like liver conditions and changes concerning the Portuguese population's access to healthcare-related services have received less attention.

AIM

To (1) Review the impact of COVID-19 on the healthcare system; (2) examine the relationship between liver diseases and COVID-19 in infected individuals; and (3) investigate the situation in the Portuguese population concerning these topics.

METHODS

For our purposes, we conducted a literature review using specific keywords.

RESULTS

COVID-19 is frequently associated with liver damage. However, liver injury in COVID-19 individuals is a multifactor-mediated effect. Therefore, it remains

unclear whether changes in liver laboratory tests are associated with a worse prognosis in Portuguese individuals with COVID-19.

CONCLUSION

COVID-19 has impacted healthcare systems in Portugal and other countries; the combination of COVID-19 with liver injury is common. Previous liver damage may represent a risk factor that worsens the prognosis in individuals with COVID-19.

Key Words: COVID-19; Healthcare systems; Liver; Portuguese individuals; Comorbidities; Clinical outcomes

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Core Tip: This review analyzes how the coronavirus disease 2019 (COVID-19) affects health care and clinical practice in Portugal and the possible causes of liver injury in COVID-19 individuals, including the direct effect of severe acute respiratory syndrome coronavirus 2 on the liver and drug-induced liver injury, and how preexisting liver diseases affect the treatment of COVID-19.

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INTRODUCTION

The coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is usually characterized by cough, headache, fever, muscle pain, general weakness, difficulty breathing, fatigue, and loss of taste and smell[1,2]. More severe cases may present pneumonia with fever, cough, dyspnea, and respiratory failure[1,2].

These symptoms may be aggravated by preexisting health conditions or comorbidities, which have been addressed in studies on the clinical characteristics and outcomes of COVID-19 individuals[2-4] published since the official declaration of the pandemic outbreak of COVID-19 on March 11, 2020[5].

Some of the most frequent comorbidities described in these studies are hypertension, diabetes mellitus, chronic obstructive pulmonary disease, cardiovascular disease, chronic kidney disease, and cancer[2-4]. In addition, despite not being among the most frequent comorbidities, the pathologies associated with the liver are hardly ever missing from the list of preexisting health conditions in COVID-19 individuals[6]. For example, a study that associated comorbidities with COVID-19 severity and hospitalization found that, concerning liver diseases, similarly to other studies[7,8], individuals with previous hepatic disorders (HD) were at a higher risk of developing symptoms when infected with SARS-CoV-2[9].

In Portugal, several studies have also focused on characterizing the clinical manifestations and outcomes of COVID-19[10-13], and some others have synthesized the clinical manifestations of COVID-19, for example, in the gastrointestinal system[14], in organs like the lungs, heart, kidney, and brain[15], or target specific population like children[16].

However, to date and the best of our knowledge, only one study has been published with a review of the impact of COVID-19 on the liver in the Portuguese population since 2020[17]. Data on individuals with COVID-19 who were also living with liver diseases and the implications and outcomes of this co-occurrence of health problems in the Portuguese population is ostensibly scarce. Likewise, the alterations in the healthcare system due to the pandemic and their association with COVID-19 and liver diseases have received little attention. Therefore, in this study, by reviewing the available research, we aimed to study the incidence of preexisting liver-associated diseases and liver injuries induced by COVID-19 in adults in Portugal and investigate the relationship of COVID-19 with liver diseases. On the other hand, we also seek to analyze how the COVID-19 pandemic affected access to healthcare systems and reflect on how societies should prepare for other pandemic crises in the future, thus contributing to the general and clinical body of knowledge on this topic.

MATERIALS AND METHODS

Literature search

The protocol used in this review included the following elements: (1) Research objective as specified in the introduction; (2) Eligibility criteria; (3) Search strategy and selection of studies; (4) Ethical considerations; (5) Quality assessment; and (6) Data extraction and analysis.

Eligibility criteria and data extraction and analysis

All the studies assessed in this review comply with the following inclusion criteria established beforehand by the authors: (1) Studies reporting original research published in Portuguese or English; (2) Targeting the Portuguese adult population, *i.e.*, 18 years old or older; (3) From any period since the official diagnosis of the first case of COVID-19 in Portugal on March 2, 2020[18]; and (4) Reporting any liver-associated clinical manifestation or outcome.

Any study that: (1) Was duplicated; (2) Was not an original research article, *i.e.*, was a case report, a conference article or an abstract-only article, a comment, a letter or editorial, an expert opinion, *etc.*; (3) Was not the final, and definite publication of the article (pre-print or accepted stage of the editorial process); or (4) Did not have data or did not refer to liver-associated diseases, was excluded.

First, the authors discussed the keywords to be used in the search, agreeing upon the following string and operators: ["Portugal" or "Portuguese patients" AND "COVID" OR "SARS-CoV-2" OR "coronavirus" AND "liver" or "hepatic condition" OR "chronic hepatitis" AND "clinical manifestations" OR "outcomes" AND "COVID-19 treatment"] to be found either in the titles, abstracts, or keywords of the articles.

The analysis began with a search of the literature available using the software *Publish or Perish*[19] to survey the databases *PubMed*, *Scopus*, *CrossRef*, *Google Scholar*, and *Web of Science*. Any study containing the string and operators mentioned before, either in the title or the abstract, was to be examined. The search was carried out on June 17, 2022, and the results of the search were exported to an *Excel* table.

A two-stage approach was used to assess the retrieved studies. First, only the titles and abstracts were scanned to determine eligibility according to the research objective and criteria. Second, the full texts of the selected papers were read and thoroughly analyzed. All the authors participated in these analyses.

Additionally, several other sources were examined to develop and elaborate on the results obtained. Some of these sources are other papers on the main topics of this review and the Portuguese National Health System (NHS), which was consulted for official information on COVID-19 and the health services at the time of the pandemic.

This study reports the review of published original research articles. Each assessed study addressed ethical specifications and approvals required by deontological practices and the law to be carried out and published. We did not recruit participants or request any information on any individual. Therefore, no formal consent or approval from any ethics committee was required.

The design of the protocol included meetings to discuss procedures and results. Cases of disagreement between the reviewers would have been resolved by resorting to two other independent researchers in the field. However, a consensus was reached about the studies to be included and excluded and the results of the analyses.

Likewise, all studies considered in this review were assessed methodologically to determine if biases were minimized or excluded. Finally, all the results are reported narratively and using tables.

RESULTS

We retrieved 804 articles from the database search. Of those, eight articles were duplicated and, therefore, eliminated. Then, the title and abstract of the 796 studies selected were screened, and 748 articles were excluded for: (1) Not referring to Portugal (n = 747); and (2) Not having the full text of the article available (n = 1) (Figure 1).

The remaining articles (n = 48) were equally distributed among the authors to assess based on the inclusion criteria. Of those, 42 articles were excluded for: (1) Not referring to the liver but instead covering comorbidities in the Portuguese population related to areas such as the cardiovascular system [20], kidney pathologies[13], respiratory system[10,21], mental health in the elderly[22], and a survey to parents on the use of healthcare services during the COVID-19 pandemic[23] (n = 27); (2) Due to lack of information to verify if the inclusion criteria were met (n = 11); (3) Not referring to data related to the liver or COVID-19 (n = 3); and (4) Referring to data on the liver but not under COVID-19 conditions (n = 1).

Finally, six studies complied with the inclusion criteria. Figure 1 shows the selection process flowchart, and the reviewed articles' characteristics are described in Table 1 of the section: *Liver diseases and COVID-19 in Portuguese individuals*.

COVID-19, healthcare, and clinical practice in Portugal

COVID-19 has greatly affected the Portuguese population's life, modifying their lifestyle, especially consumption habits[24]. Socialization with friends and family was reduced due to measures such as

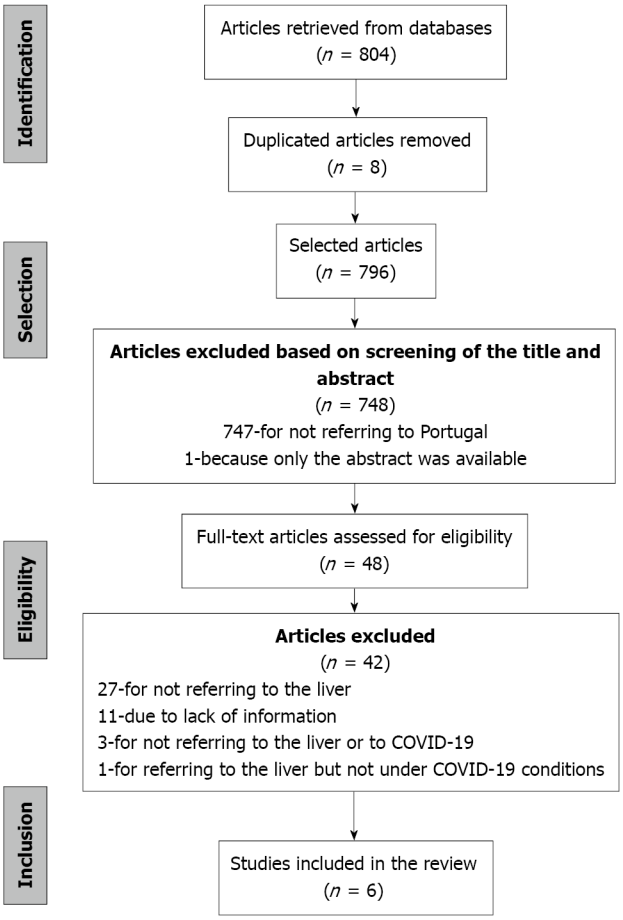
Table 1 Coronavirus disease 2019 studies in Portuguese individuals with preexisting liver disease

Ref.	Breadth and type of study	Analyzed individuals (*N*)	Data source	Period of data collection	Age range (yr)	Preexisting liver disease (%)	Study findings
[63]	National Retrospective (observational) study	20.293	SINAVE (DGS)	January 1-April 21, 2020	[0 to > 86]	0.53%	Risk of mortality: (1) Higher in the male gender; (2) Higher in older age groups; and (3) Increases with the presence of comorbidities
[61]	National Retrospective observational study	36.244	DGS	March 2–June 30, 2020	[18 to > 80]	0.57%	The presence of simultaneous diseases is significantly associated with negative outcomes in individuals with COVID-19
[60]	National Retrospective cohort study	38.545	DGS	March 2–June 30, 2020	[0 to > 70]	0.60%	Risk of hospitalization: (1) Lower in females; (2) Increases with age; and (3) Increases with the presence of comorbidities
[62]	National Retrospective cohort study	20.293	DGS	March 1-April 28, 2020	[0 to > 90]	0.50%	Risk of hospitalization, ICU admission, and death: (1) Higher in males; (2) Increases with age; and (3) Increases with the presence of comorbidities
[64]	National Retrospective (observational) study	6.701	SICO DGS)	The year 2020	[0 to > 0]	No information available	The risk of hospitalization and lethality is higher in males and increases with age
[59]	A retrospective cohort study in a university hospital center	317	University Hospital Center of Porto (CHUP)	March 2-May 4, 020	[18 to 5]	4.42%	Hepatic enzyme changes in COVID-19 individuals were frequent but mostly mild; AST, unlike ALT, was associated with worse clinical outcomes, such as the severity of COVID-19 and mortality

All results are represented in frequency (N, number of individuals) and percentage (%). General Health Directorate of Portugal–DGS (Direção geral de Saú de de Portugal)/National e-Death Certificates Information System–SICO (Sistema de Informação do Certificados de Óbito) (SICO)/National System for Epidemiological Surveillance–SINAVE (Sistema Nacional de Vigilância Epidemiológica). COVID-19: Coronavirus disease 2019; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ICU: Intensive care unit.

social distancing and restriction of access to many public services adopted to break the COVID-19 transmission chains. In Portugal, the National Health Service also enforced the suspension of all elective and non-urgent health care[25]. This measure had long-term consequences, such as an impact on the waiting lists for hospital medical specialties for first evaluation or other complex procedures/treatments (for example, surgeries and physiotherapy). During that period, all laboratories were exclusively dedicated to testing for COVID-19, reducing non-urgent activities and re-organizing their tasks.

Primary health services were also impacted, and many appointments were canceled or modified. A Portuguese study by Vieira *et al*[26] investigated all medical appointments in a primary care unit in the central region of Portugal from March 1 to July 31, 2020, and in the same period of 2019 and 2018. The authors observed that, contrary to what was expected, there was an increase (about 14% when compared with 2018 and 4% when compared with 2019) in the number of medical appointments in 2020 compared with the same period of 2018 and 2019. However, most of these appointments were teleconsultations performed by e-mail or phone compared with previous years (71.6% in 2020, 42.6% in 2019, and 35.2% in 2018). On the other hand, there was also a decrease in most follow-up and routine appointments and an increment in psychiatric diagnoses, mainly anxiety-related disorders[26]. Although this study demonstrated that the total number of medical appointments increased in 2020, mostly due to teleconsultations, this increase may not have reflected the general situation in other primary health units across the country. One reason for this hypothesis is that the central region in Portugal was less affected by the COVID-19 pandemic in the period when the study was performed. If the same study had been replicated in the northern region, the results would have been different. Data from the NHS revealed that in March 2020, a reduction of 16% in speciality medical appointments in public hospitals was observed compared with the same period of 2019. This negative variation was even greater in the following months, April and May, when it increased to 25% and 31%, respectively[27]. The first specialty medical appointment in the hospital was also reduced in these months in 2020 when compared with 2019. However, telemedicine appointments (not face-to-face appointments) increased [27]. The number of scheduled and urgent surgeries also decreased in these months by 15%, 38%, and 32% compared with March, April, and May of 2019, respectively. The emergency cases arriving at the hospital also decreased during this period[28].



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Figure 1 Flowchart of the studies selection process.

When the data from January to July 2019 and 2020 were compared, a reduction in face-to-face appointments, an increase in telemedicine, and a reduction in medical tests were observed. Regarding the latter, pathological anatomy tests decreased by 36%, and tests concerning surgery medical specialties, which may study the liver, decreased by 32%[27].

More face-to-face nursing consultations occurred in 2019 compared with 2020, and the same was observed regarding face-to-face medical consultations. However, regarding cancer screening, in 2020 compared with 2019, fewer women got mammographies in the previous 2 years, fewer women updated their Pap smears, and fewer individuals got their colorectal cancer screening[29].

Portugal was not the only country whose health services were affected by the COVID-19 pandemic. Other European and non-European countries also suffered adjustments and had similar results to those observed in Portugal in primary care health services during COVID-19[30]. In this regard, some authors have proposed lessons on the COVID-19 pandemic for primary care professionals worldwide[31]. The main aspects that these authors consider essential for health services to learn and adapt are: (1) Rapidly changing and innovating by redirecting patient flow like primary care; (2) Maintaining access to primary care and managing all health problems that impact the local population; and (3) Collecting and disseminating information that is sufficiently fitted for the purpose[31].

The alterations in the operation of laboratories were studied mainly in the laboratories dedicated to diagnosing malignant pathologies.

In the cytopathology laboratories of 23 countries (including Portugal), the number of samples analyzed during 2020 compared with the same period in 2019 was reduced by 45.3%[32]. While the number of samples from the cervicovaginal tract, thyroid, and anorectal region decreased significantly, and the percentage of samples from the urinary tract, serous cavities, breast, lymph nodes, respiratory tract, salivary glands, central nervous system, gastrointestinal tract, pancreas, liver, and biliary tract increased[32].

Also, lab facilities were adapted to work in COVID-19-free environments. Glasbey *et al*[33] compared hospitals with and without COVID-19-free surgical pathways to investigate if implementing these pathways could be associated with lower postoperative pulmonary complication rates. This study demonstrated that COVID-19-free surgical pathways should be established within available resources to provide safer elective cancer surgery during current and future COVID-19 outbreaks. As health

providers resume elective cancer surgery, they should prevent harm by investing in dedicated COVID-19-free surgical pathways tailored to local resources[33].

COVID-19 and liver disease

The SARS-CoV-2 infection is frequently associated with acute liver injury (ALI), evidenced by increased transaminases in individuals with and without prior liver disease. Elevated transaminases affected up to 50% of SARS-CoV-2 infected subjects and correlated with the severity of the disease[34].

Transaminase elevation during COVID-19 is mostly mild and reversible (*i.e.*, < 5 times higher than the normal limit). Severe ALI is uncommon, occurring in 0.1% on admission and 2% during hospitalization, with alanine aminotransferase (ALT) values 20 times higher than the normal limit. These individuals had abnormal liver tests before being hospitalized[35].

ALI reflected by increased transaminases and bilirubin was related to disease severity and poor outcome of COVID-19[36]. Increases in gamma glutamyl transferase (γGT) and alkaline phosphatase (AP), also liver-related enzymes, are less observed and found more frequently in the later course of the disease. In contrast, moderate increases in aspartate aminotransferase (AST), ALT, and bilirubin are very common features in COVID-19[37].

Cholangiopathy, which is focal or extensive damage to bile ducts due to an impaired blood supply, has been reported as a late complication of severe COVID-19, and some persons have developed progressive biliary injury and liver failure[38,39]. In a study with 2047 patients hospitalized for COVID-19, 12 (approximately 0.58%) with severe COVID-19 developed a syndrome of cholangiopathy characterized by cholestasis and biliary tract abnormalities[38]. The mean time from COVID-19 diagnosis to cholangiopathy was 118 d. Imaging findings included inflammation, beading, stricturing, and dilation of the biliary tree.

Angiotensin-converting enzyme 2 (ACE2) receptor and the SARS-CoV-2 co-receptor transmembrane serine protease 2 seem to be the key host receptors for this virus and are expressed on diverse liver cells such as hepatocytes, immune cells, cholangiocytes, and liver progenitor cells. The levels of ACE2 are very low in liver cells, accounting for 2.6% of the total number of cells, but it is highly expressed in bile duct cells (59.7%)[40] (Figure 2).

The liver may undergo several injuries during the infection period. According to several authors, these aggressions may result from: (1) A direct virus attack on the different types of liver cells; (2) A magnified immune response (cytokine storm); and (3) Toxicity of the drugs used to treat COVID-19 symptoms. Whether one or the sum of these insults contributes to liver disruption has yet to be clarified. The 'cytokine storm' that occurs in COVID-19 is common to other viral infections and involves a massive acute phase reaction that results from the release of huge amounts of proinflammatory cytokines, such as interleukin (IL)-1, tumor necrosis factor, and IL-6, as well as an increased concentration of C reactive protein and ferritin. The bulk of these cytokines is released by macrophages and monocytes, assumed to be the main players of the 'cytokine storm' that occurs in SARS-CoV-2 infection [41,42] (Figure 2).

Whether individuals with chronic liver disease (CLD) are more susceptible to COVID-19 is uncertain. CLD, in the absence of immunosuppressive therapy, is not known to be associated with an increased risk of acquiring COVID-19[36]. However, the liver may be susceptible to SARS-CoV-2 because of ACE2 receptors in the biliary and liver epithelial cells[40].

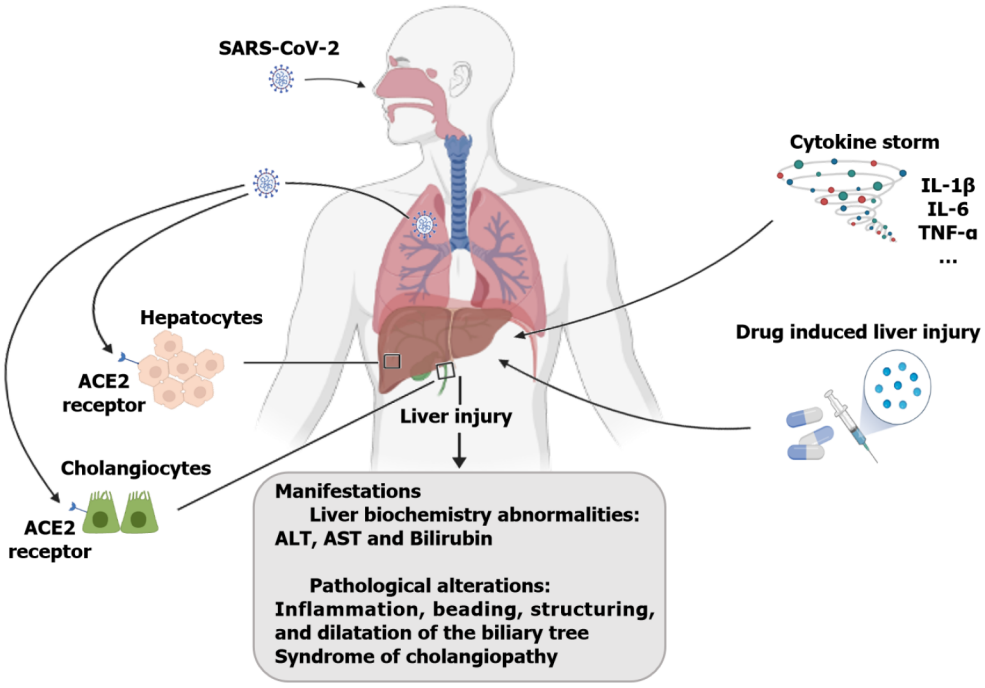
Data have suggested that preexisting liver disease was associated with worse outcomes in individuals with COVID-19. In a study of 2780 persons with COVID-19, those with CLD (n = 250) had higher rates of mortality as compared with those without liver disease (12% *vs.* 4%; risk ratio: 2.8, 95%CI: 1.9-4.0)[9,43,44]. Similarly, research carried out in Bangladesh on the relation of comorbidities, the severity of COVID-19, and hospitalization found that 43.6% of the individuals with HD (n = 39 in a sample of 1025) had more serious, *i.e.*, moderate to severe, symptoms of COVID-19[9]; in another study in the same country on the relation of comorbidities and health outcomes in COVID-19 persons, the authors concluded that several comorbidities, including liver diseases, influence the severity of outcomes and fatality in these individuals[6].

Subsequently, a large French retrospective cohort of > 259000 individuals with COVID-19, including > 15000 with preexisting CLD, demonstrated that those with decompensated cirrhosis were at an increased adjusted risk for COVID-19 mortality[45].

Acute-on-chronic liver failure is also well recognized, being reported in up to 12%–50% of people with decompensated cirrhosis and COVID-19. Although acute hepatic malfunction induced by the SARS-CoV-2 virus may aggravate decompensated cirrhosis, in most cases, this liver aggravation did not contribute to the death of individuals, which occurred mostly due to respiratory failure (71%), followed by liver-related complications (19%)[44].

Hepatic conditions affecting COVID-19 treatment

CLD is considered a serious problem worldwide. Liver injuries like cirrhosis, chronic viral hepatitis, alcohol-related liver disease, non-alcoholic fatty liver disease, and autoimmune hepatitis affect approximately 50 thousand people in Portugal, most of which are still undiagnosed[46]. Therefore, it is important to carefully evaluate how different existing liver diseases influence liver damage in persons with COVID-19[47,48].



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Figure 2 Mechanisms that may lead to liver injury in individuals with coronavirus disease 2019. An infection by severe acute respiratory syndrome coronavirus 2 may result in liver injury due to viral entry through angiotensin-converting enzyme 2 receptors on hepatocytes and cholangiocytes, pharmacological treatment, or an exaggerated immune response. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; ACE2: Angiotensin-converting enzyme 2; IL: Interleukin; TNF-α: Tumor necrosis factor-α.

Individuals with chronic hepatitis B (CHB) treated with nucleoside analogs for long periods are in a phase of immune tolerance. Therefore, the virus that causes hepatitis is inhibited. However, when infected with SARS-CoV-2, they can suffer severe liver injury[48].

Previous studies[47,48] have shown that for persons with hepatitis B undergoing antiviral therapy, stopping drug administration may lead to reactivation and replication of the hepatitis B virus (HBV) after treatment against SARS-CoV-2 infection is done. It has also been previously reported[47,48] that CHB patients infected with SARS-CoV-2 may take longer to clear the virus from their bodies. Zha *et al* [49] found a correlation between chronic HBV infection and the time required to eliminate SARS-CoV-2 from the individual. This association may have to do with T-cell dysfunction in people with chronic HBV infection fighting other viruses, but the link between them requires future investigations.

Other clinical studies have noted that the combination of lopinavir and ritonavir in the treatment of individuals infected with SARS-CoV-2 may increase liver damage in those with hepatitis B or hepatitis C[47], so the use of these drugs in the treatment of COVID-19 may promote or worsen liver injury in people with preexisting liver disease[47,48].

Another risk factor that may be related to disease severity and mortality and is common in persons with COVID-19 is dysfunction in the immune system[47,48]. In persons with autoimmune hepatitis who have been infected with SARS-CoV-2, special attention should be paid to the effect of glucocorticoid administration on the prognosis of the disease[47,50]. Considering the high level of ACE2 receptor expression in bile duct cells, individuals with cholangitis infected with SARS-CoV-2 may see their cholestasis worsen, resulting in increased levels of AP and γGT[48]. Moreover, persons with cirrhosis and liver cancer with systemic immunodeficiency might be more susceptible to SARS-CoV-2 infection, as their immune function is relatively lower[47,50].

For individuals with COVID-19 and mildly abnormal liver function, the administration of anti-inflammatory and hepatoprotective drugs is generally not required[48]. Still, if necessary, the initial disease should be treated with antiviral and supportive drugs that inhibit viral replication, reduce inflammation, and enhance immunity. Additionally, the pre-emptive application of liver-protective and liver enzyme-lowering drugs is not recommended[47].

In people with COVID-19 and acute liver damage, clinicians should analyze and judge the most probable cause of liver injury and then take the appropriate action[47,48]. Routinely, these persons should be treated with anti-inflammatory, hepatoprotective drugs, and jaundice-reducing agents such as polyene phosphatidylcholine, glycyrrhizic acid, bicyclol, and vitamin E[48]. In critically ill patients, the therapy should be chosen according to liver function injury, the types of drugs administered should not be more than two, and the dosage should not be too high to avoid worsening liver damage and drug interactions[49].

In persons with COVID-19 suspected of having liver damage caused by drugs, stopping or reducing the amount of the drug should be considered. Besides that, liver function indicators should be closely monitored to prevent acute liver failure[47,48]. Respiratory and circulatory support should be reinforced in individuals with critical and severe COVID-19 disease with liver damage since the clinical status should be caused by cytokine storms and microcirculation ischemia and hypoxia[47]. If necessary, extracorporeal membrane oxygenation should be performed to improve the blood oxygen saturation of patients[47].

In this way, control and prevention of the inflammatory response in the early phase of the disease not only helps to reduce nonspecific inflammation in the liver but also prevents the occurrence of a systemic inflammatory response as a way to mitigate the likelihood of mild disease developing into severe or critical illness[47,48].

The main drugs used to treat COVID-19 in individuals with severe or strong symptoms, *i.e.*, hydroxychloroquine[51,52], lopinavir/ritonavir[53], and remdesivir[54-58], have adverse effects in the liver (Table 2). Such effects limit the use of these drugs in persons with previous liver diseases who may experience an aggravation of COVID-19 symptoms (as explained before).

Liver diseases and COVID-19 in Portuguese individuals

The impact of SARS-CoV-2 infection on the liver and the hypothesis that having liver injury may represent a risk factor for the worsening of COVID-19 prognosis have been addressed by several authors[47,48,59].

In Portugal, an epidemiological study by Tenreiro *et al*[60], in a sample of 35117 individuals with COVID-19, demonstrated that the risk of hospitalization increases with the presence of comorbidities and that preexisting pathologies, such as CLDs, are associated with worse prognosis in people with COVID-19 (Table 1). In agreement, Froes *et al*[61] (2020) and Ricoca Peixoto *et al*[62] (2021) observed that the presence of concurrent comorbidities is significantly associated with negative outcomes when the person is infected with SARS-CoV-2 (Table 1).

In the different studies (Table 1), it was also possible to observe that in the Portuguese population, the risk of hospitalization and lethality associated with COVID-19 in infected individuals is higher in males and increases with age[60-64]. Furthermore, Musa[65] also noted that people at high risk of severe COVID-19 are generally older and/or have associated complications such as diabetes, hypertension, cardiovascular disease, and liver disease.

However, the existing literature has shown contradictory data. Although some reports have shown that persons with comorbidities, such as liver failure, had longer hospitalization lengths and were at a higher risk of progressing to severe disease[58,66], which is in line with the data found in Portuguese individuals, other studies have demonstrated that the COVID-19-related liver injuries are usually temporary and mild, with minor clinical significance[59,67].

Liver biochemical indicators, mainly shown by abnormal ALT and AST levels complemented by a slight increase in total bilirubin levels and prolonged prothrombin time, have also been used as a predictor of the severity and prognosis of COVID-19 patients[47,59,68,69].

In a study performed by Guan *et al*[68] (2020), which included 1099 patients, elevated AST levels were observed in 18.2% of patients with non-severe disease and 39.4% with severe disease. In addition, the proportion of patients with abnormal ALT in severe cases (28.1%) was higher than that in mild cases (19.8%)[68]. Other studies, however, have found different results. Wang *et al*[70] and Wu *et al*[50] reported no significant differences in liver function tests when comparing severe and mild/moderate patients.

In the Portuguese literature, there is still scarce information regarding the global etiology of preexisting liver disease in individuals with COVID-19. Garrido *et al*[59], in a study of 317 individuals with COVID-19 at the University Hospital Center of Porto, observed that at the time of hospitalization, 50.3% of patients had abnormal changes in liver enzymes, with 41.5% showing elevated levels of aminotransferases. AST and AP were associated with the severity of COVID-19, intensive care unit (ICU) admission and increased mortality. In contrast, ALT and γGT were associated with patient admission to the ICU[59]. In the same study, the authors reported that of the 14 patients with CLD, 21.4% had a critical level of COVID-19, the average duration of disease was 36.6 d, hospitalization 11.5 d, and mortality 28.6%, increasing to 66.7% in patients with liver cirrhosis[59].

Portugal participated in the European study from the European Hematology Association Survey (EPICOVIDEHA) that aimed to investigate the characteristics of hematological malignancies in individuals developing COVID-19 and analyzed predictors of mortality[71]. The authors concluded that increased age, active disease, chronic cardiopathy, liver disease, renal impairment, smoking history, and ICU were significantly associated with higher mortality[71].

Recently, we published a study on the Portuguese population, with more than 2000 participants, reporting the medical and legal implications of COVID-19 in life habits[24]. We observed that the subjects increased alcohol consumption, a change that can contribute to liver diseases. On the other hand, we also analyzed all participants' comorbidities, and none indicated having a liver disease (unpublished results). It is possible that in some cases, the individuals do not even know that they have liver malfunction, or the liver disease is discovered after the SARS-CoV-2 infection and treatment.

Table 2 Drugs used in the treatment of coronavirus disease 2019 that have adverse effects on the liver

Drug(s)	Mechanism of action	Adverse effects on the liver	Ref.
Hydroxychloroquine	Increases intracellular pH and inhibits lysosomal activity in antigen-presenting cells	Liver failure	[51,52]
Lopinavir/Ritonavir	Inhibition of 3-chymotrypsin-like protease	Hepatoxicity	[53]
Remdesivir	Inhibits viral transcription and replication by blocking the RNA polymerase enzyme	Increased aminotransferase levels	[54-57,70]

However, as concluded in other reports[58,59,68], it remains unclear whether these changes in laboratory tests are associated with a worse prognosis in individuals with COVID-19 because the cause of these alterations could be explained by several factors like the virus or the percussion of a severe inflammatory response from a liver injury such as hypoxic hepatitis, drug toxicity, immune response, and alterations in the gut vascular barrier and microbiota[47,48,59].

DISCUSSION

We surveyed several databases and screened several papers to review the clinical manifestations and outcomes of Portuguese individuals with COVID-19 and liver injury. However, only six studies reported original observational and retrospective research on the Portuguese adult population concerning liver-associated clinical manifestations or outcomes and COVID-19.

Because of the relatively small number of articles analyzed in this review, further studies were considered to develop and elaborate on the results obtained. Data from the Portuguese NHS on COVID-19 was also included. In this second round of literature search, we found information about the implications of COVID-19 in healthcare. Therefore, we broadened the scope of this review, although this was not initially planned.

We examined topics essential to understanding the relationship between COVID-19, liver injury, and patients' clinical outcomes. We discussed: (1) How COVID-19 affected health care and clinical practice in Portugal; (2) The relationship between COVID-19 and liver diseases; (3) Hepatic conditions that affect the treatment of COVID-19; and (4) What is known regarding liver diseases and COVID-19 in Portuguese individuals.

Our results demonstrated the implications of the COVID-19 pandemic in Portugal's health care and clinical practice. We observed that the number of face-to-face consultations decreased during the pandemic, specifically, during the first semester, of 2020, compared with the same period of the last 2 years (2018/2019). However, virtual medical assistance, *i.e.*, medical and nurse teleconsultations, increased. Besides these changes in medical appointments, we also observed adjustments in the activity of diagnostic laboratories. A European study involving Portugal demonstrated that the percentage of analyzed samples increased during the pandemic, specifically from the liver, urinary tract, serous cavities, breast, lymph nodes, respiratory tract, and others. On the contrary, the percentage of analyzed samples of the cervicovaginal tract, thyroid, and anorectal region decreased[32].

While exploring the role of liver injury and COVID-19, we observed that COVID-19 affects liver activity, changing the enzymatic activity[37]. These enzymatic alterations were observed in half of the infected individuals[34]. On the other hand, preexisting liver disease was associated with worse outcomes in persons with COVID-19. For this reason, in Portugal, having a liver disease (chronic disease) was enough to be included in priority groups for vaccination during the first campaign of the Portuguese NHS.

The liver and COVID-19 are interrelated. On the one hand, liver function affects COVID-19 status. On the other hand, drugs such as lopinavir, ritonavir, hydroxychloroquine, and remdesivir, used to treat COVID-19, may cause adverse effects on the liver and affect its function (Table 1).

According to our search, the clinical outcome in Portuguese individuals with COVID-19 was aggravated by liver injury, old age, and other comorbidities leading to treatment in intensive care and increased mortality.

CONCLUSION

COVID-19 was a pandemic that had many implications for people's health, life quality, and lifestyle and their access to public or "social" services, such as primary healthcare services. The global dimension of COVID-19 consequences is almost unmeasurable. However, our lives have changed, and these alterations may be permanent. Not so much time ago, before the COVID-19 pandemic, teleconsultations were unthinkable. During the pandemic, telemedicine became a form of access to medical assistance.



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Figure 3 Alterations in people's quality of life and healthcare systems due to coronavirus disease 2019.

Furthermore, all health systems had to adapt during COVID-19, prioritizing healthcare. According to this evidence, we conclude that societies should define strategies to be implemented during pandemic situations to ensure that all individuals have guaranteed access to health care (Figure 3). In Portugal, we observed that the alterations that occurred in the healthcare system during the pandemic seem to have been effective in providing care to the population.

Concerning the relationship between liver diseases and COVID-19, we conclude that COVID-19 may alter the function of the liver. The worsening of the COVID-19 status depends on the age of the individuals and the presence of other comorbidities, albeit the papers analyzed in this review do not discuss if the liver function is age-dependent. Moreover, the drugs used to treat COVID-19 have adverse effects on the liver and affect liver function.

ARTICLE HIGHLIGHTS

Research background

Coronavirus disease 2019 (COVID-19) may be asymptomatic or cause mild to severe symptoms exacerbated by preexisting health conditions like liver diseases. However, the relation between hepatic diseases and the effect and prognosis of COVID-19 individuals is unclear, and information concerning the Portuguese population is scarce.

Research motivation

To understand how hepatic disorders may have been affected by COVID-19 and how this infection impacted the Portuguese healthcare system.

Research objectives

To investigate the association between COVID-19 and liver diseases and their impact on the prognosis of patients and explore the effect of COVID-19 on healthcare systems.

Research methods

A review of the relevant literature was performed.

Research results

People with liver diseases who were COVID-19-positive had a worse prognosis than individuals without hepatic diseases. Due to COVID-19, most countries altered the normal activity of healthcare systems, increasing teleconsultations.

Research conclusions

The interaction between liver diseases and COVID-19 in people's prognosis is unclear, but there is evidence of its existence. As observed in other countries, the normal activity of the national healthcare system in Portugal was modified due to the pandemic situation created by COVID-19.

Research perspectives

To promote the development of studies about the interaction and relation between liver diseases and COVID-19 and generate critical thinking about how healthcare systems should function in case of a

pandemic.

FOOTNOTES

Author contributions: Fernandes S and Sosa-Napolskij M contributed equally to this work; Fernandes S, Sosa-Napolskij M, Lobo G, and Silva I designed the research study, performed the research, analyzed the data, and wrote the manuscript; all authors have read and approved the final manuscript.

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Country/Territory of origin: Portugal

ORCID number: Isabel Silva 0000-0001-7244-4792.

S-Editor: Fan JR
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P-Editor: Fan JR

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Editorial Board of *World Journal of Gastroenterology*, Chun-Feng Qu, MD, PhD, Director, Professor, Department of Immunology, National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, No. 17 Panjiayuan South Lane, Chaoyang District, Beijing 100021, China. quchf@cicams.ac.cn

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EDITORIAL

COVID-19-induced transaminitis and hyperbilirubinemia: Presentation and outcomes

Zeinab Nabil Ahmed Said, Safinaz Adel El Habashy, Samy Zaky, ESCMID Study Group for Viral Hepatitis

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Zeinab Nabil Ahmed Said, Department of Medical Microbiology and Immunology, Faculty of Medicine (For Girls), Al-Azhar University, Cairo 11754, Nasr City, Egypt

Safinaz Adel El Habashy, Department of Pediatrics, Ain Shams University, Cairo 11391, Abbaseia, Egypt

Samy Zaky, Department of Hepato-gastroenterology and Infectious Diseases, Faculty of Medicine (For Girls), Al-Azhar University, Cairo 11754, Egypt

Corresponding author: Zeinab Nabil Ahmed Said, PhD, Additional Professor, Medical Microbiology and Immunology, Faculty of Medicine (For Girls), Al-Azhar University, Elshenawy St., Cairo 11754, Nasr City, Egypt. znabil58@yahoo.com

Abstract

The risk of liver injury in patients with coronavirus disease 2019 (COVID-19) infection is quite evident. Furthermore, liver function test abnormalities are still detected in COVID-19 patients despite the development of antivirals and the availability of several types of vaccines. This editorial describes liver involvement during COVID-19 infection in patients with or without preexisting liver injury, such as chronic liver disease, to elucidate COVID-19-induced liver function abnormalities and their severity, pathophysiology, clinical manifestations, and clinical and laboratory outcomes. We also discuss the effect of vaccination against COVID-19 to better understand host factors, such as age, gender, and race, on the incidence and severity of liver dysfunction at initial presentation and during the illness. Finally, we summarize the results of relevant meta-analyses published to date and highlight the importance of adequate liver function monitoring in the current climate of the overwhelming COVID-19 pandemic.

Key Words: COVID-19; SARS-CoV-2; Liver injury; Transaminases; Hyperbilirubinemia; Pathophysiology

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Core Tip: Recent evidence confirmed coronavirus disease 2019-induced liver function test abnormalities in patients with or without preexisting liver injury. Understanding the mechanism and recognizing the clinical picture, as well as identifying the risk factors for developing such abnormalities, will pave the way for early diagnosis and better management of such cases.

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INTRODUCTION

The continuing evolution of coronavirus disease 2019 (COVID-19) has led to the identification of a wide spectrum of associated symptoms, ranging from asymptomatic disease to severe manifestations that resulted in acute respiratory distress syndrome, respiratory failure, or multiple organ dysfunctions with risk of thrombosis and death[1,2]. Accumulating evidence indicates an association between COVID-19 infection and liver function test (LFT) abnormalities, and there have been many reports of liver injury, even in those without pre-existing liver disease[3-5]. It was shown that COVID-19 binds to angiotensin-converting enzyme 2 (ACE2) receptors (part of the renin–angiotensin system) to gain entry and damage the target organ[2]. Since ACE2 receptors are found in both bile duct epithelial cells (cholangiocytes) and liver cells (hepatocytes)[4], the liver is a potential target for direct infection. COVID-19 liver infection is related to disease severity and older age[5], and LFTs usually reveal a cholestatic or hepatocellular pattern[6]. Thus, a more detailed understanding of host factors including underlying comorbidities, in addition to adequate monitoring of patients with liver damage, is mandatory in the current overwhelming COVID-19 pandemic[7].

INCIDENCE OF COVID-19-INDUCED LIVER DYSFUNCTION

Sun *et al*[8] defined COVID-19-related liver injury as any liver damage occurring during disease progression and treatment in patients with or without pre-existing liver disease. The reported incidence of LFT abnormalities observed with COVID-19 is variable. A recent meta-analysis of 107 studies consisting of 20874 COVID-19-positive patients reported the pooled incidence of elevated liver enzymes at presentation as 23.1%[9]. A similar frequency was reported in another meta-analysis, where abnormal aminotransferase levels were present in 24% of 17776 patients[10]. Most enzyme elevations associated with COVID-19 infection are transient and self-limited[8]. Notably, elevated aminotransferase levels were reported in 14%–58% of hospitalized patients with COVID-19[4,11]. Additionally, the pooled prevalence of elevated alanine transaminase (ALT), aspartate transaminase (AST), and total bilirubin were evaluated as 21% (14%–29%),18% (13%–25%), and 6% (3%–11%), respectively[12]. Males are at high risk of getting acute liver injury related to COVID-19 than females since it was reported that bilirubin, ALT, alkaline phosphatase (ALP), and gamma-glutamyl transferase values were higher in male patients with COVID-19[13,14]. Regarding age, a meta-analysis by Kulkarni *et al*[10] revealed that the incidence of elevated liver enzymes in children (> 10 years) was 17.8% [95% confidence interval (CI): 9.9–29.8] among 283 patients, meanwhile, in adults it was 24.1% (95%CI: 20–28.8) among 12756 patients. There was also a significant difference in the prevalence of liver injury among different races and ethnicities. On adjusted analyses, white patients were less likely to develop liver injury compared with Asian patients (OR: 1.65, 95%CI: 1.37–2.02) and multiracial patients (OR: 1.65, 95%CI: 1.37–2.02). Those with a non-Hispanic ethnicity had a lower association with sustaining liver injury (OR: 0.77, 95%CI: 0.75–1.03)[15].

PATHOPHYSIOLOGY

The detailed mechanism of liver injury in COVID-19 infection remains unclear. Several possibilities involving a combination of direct viral-mediated effects due to viral replication within hepatocytes[8,9,16] and the viral-induced cytokine storm have been postulated[8,17]. Remarkably, severe acute respiratory syndrome coronavirus-1 RNA was detected in liver tissue from SARS-infected patients, although viral inclusions were not detected under electron microscopy[18]. Viral entry occurs through ACE2 receptors, which are expressed on many cell types, including hepatocytes and cholangiocytes[9,19]. Increased ACE2 expression in cholangiocytes (59.7% of cells) and, to a lesser extent, hepatocytes

(2.6% of cells) confirms that severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection alters liver function by direct cytotoxicity due to continuous viral replication[20]. However, severe SARS-CoV-2 infection results in a clinical state resembling sepsis due to the massive release of cytokines, which may progress to apoptosis and necrosis of infected cells, resulting in multiorgan failure late in the course of the disease[21]. Immune-mediated injury is supported by a marked rise in serum ferritin, lactate dehydrogenase, interleukin (IL)-2, and IL-6[6,19]. Pneumonia-associated hypoxia or ischemic hepatitis due to prolonged hypotension/shock is also speculated[5,6,8,9,16]. Exposure to hepatotoxic agents must be considered since drug-induced hepatotoxicity varies with age, sex, and race [22]. Antiviral agents directed against COVID-19 (*e.g.*, lopinavir or ritonavir), antibiotics used against bacterial infections, antipyretics, anticoagulants, and steroids may also cause liver function deterioration [23,24]. Additionally, underlying liver disease is considered a risk factor.

CLINICAL/LABORATORY MANIFESTATIONS

COVID-19-related liver injury may manifest as hepatobiliary symptoms and elevated liver enzymes. Among patients with COVID-19, liver symptoms are not atypical and may be present without any respiratory symptoms. Furthermore, hepatic symptoms are associated with worse clinical outcomes and an increased risk of mortality[25]. The incidence of a worse clinical outcome is high in hospitalized COVID-19 patients who suffer from jaundice. Additionally, the intensive care unit (ICU) admission rate is approximately 2.5 times higher for patients with hepatic jaundice ($P < 0.001$), mainly due to complicated bacterial sepsis or severe systemic inflammation[26].

Patients with COVID-19, both with and without pre-existing liver disease, may have elevated aminotransferase levels. A mixed pattern of both hepatocellular and cholestatic affection, without significant liver synthetic dysfunction has been reported, although ACE2 receptors are more frequently expressed on cholangiocytes than hepatocytes[4,27]. There is usually a greater elevation of AST than ALT levels, and this pattern has been associated with disease severity. Additionally, both ALT and AST are more usually elevated than bilirubin or ALP[28,29]. Sun *et al*[8] categorized the degree of liver damage as mild if ALT was elevated < 2 × upper limit of normal (ULN), moderate if 2 × < ALT < 5 × ULN, and severe if ALT > 5 × ULN. However, lower AST/ALT ratios may be more specific for hepatic injury[6]. A retrospective study evaluating the levels of hepatic enzymes of 1827 COVID-19 patients at admission and during hospitalization demonstrated abnormal levels of AST (66.9%), ALT (41.6%), and ALP (13.5%) at admission with peaks of AST (83.4%), ALT (61.6%), and ALP (80%) during hospitalization[30]. Another retrospective cohort study on 230 Covid-19 positive patients showed that the prevalence of abnormal liver enzymes among those with severe COVID-19 infection were as follows: AST (77%), ALT (49%), gamma glutamyl transpeptidase (GT) (37%), and ALP (12%). A severe COVID-19 infection was more likely present in patients with abnormal levels of AST ($P = 0.015$), gamma GT ($P = 0.022$), and ALP ($P = 0.03$)[31].

Regarding age, children appear to have a milder illness with significantly less need for inpatient admission or respiratory support and are less likely to have the multiple comorbidities present in older adults. Hepatitis is common in children with multisystem inflammatory syndrome and is associated with a more severe presentation and persistent elevation of LFTs in many patients[32]. Furthermore, older patients are more likely to develop more severe COVID-19 and at greater risk of abnormal liver function. The latter is more common in patients with severe or critical presentations of COVID-19[5]. In pregnant women with COVID-19, observational studies showed an increased prevalence of preeclampsia and hemolysis, elevated liver enzymes and low platelet (HELLP) syndrome. Despite a possible pathophysiology linkage between COVID-19 and HELLP syndrome, the evidence on temporality to prove a causal association between SARS-CoV-2 infection and HELLP syndrome is still insufficient[33].

A cohort study reported that acute liver injury with a hepatocellular pattern was common in patients who tested positive for SARS-CoV-2 but was usually mild[16]. However, 6.4% of patients had a severe liver injury with a severe disease course, where elevated AST levels may be indirect indicators of multiorgan involvement[34]. There is also a recent case report of a young male with COVID-19 who suffered from acute icteric hepatitis with a marked rise in bilirubin and liver transaminase levels without any respiratory symptoms[6]. Another case report found that COVID-19 infection could be a risk factor or comorbidity of acute liver failure, with only isolated hyperbilirubinemia indicating liver involvement[17]. Moreover, severe infections with COVID-19 followed by death were more often associated with hypertransaminasemia and high bilirubin levels compared with mild and moderate infections[14,35]. Patients with severe liver injury were more likely to need ICU-level care, intubation, and renal replacement therapy and showed a greater risk of in-hospital mortality[6,16,36]. A high bilirubin level and liver stiffness (measured using shear wave elastography) have been reported as correlated with more severe outcomes[37-39]. Liver injury and failure are frequently observed in critically ill cases, and their occurrence is associated with high morbidity and mortality[40-42]. Recently a potential link between Omicron variant infection and severe hepatitis of unknown etiology in children was observed, where it was postulated that previous infection or co-infection with SARS-CoV-2

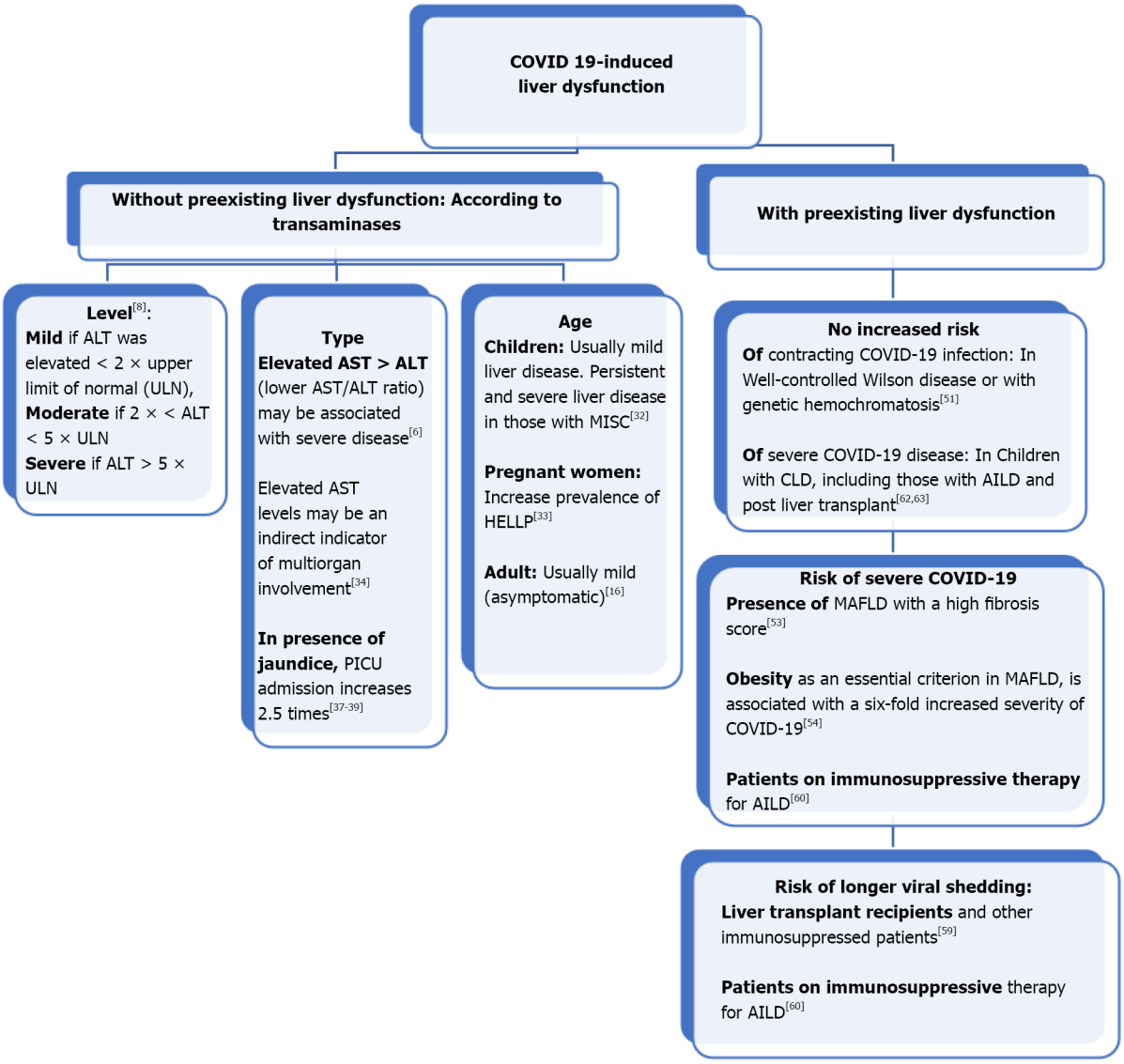
increases the susceptibility to adenovirus infection[43]. Figure 1 summarizes the clinical and laboratory presentation of COVID-19-induced liver dysfunction.

COVID-19 IN- PATIENTS WITH PRE-EXISTING LIVER DISEASE

The impact of COVID-19 on chronic liver disease (CLD) is variable. Several studies have reported that patients with CLD, regardless of its etiology, may be at higher risk for severe illness from COVID-19[44-47]. A systematic review of 40 studies with 908032 participants (most of them from China and United States) showed that COVID-19 cases with CLD had significantly higher chance of having a severe form of COVID-19 (pooled OR: 2.44; 95%CI: 1.89–3.16) and death (pooled OR: 2.35; 95%CI: 1.85–3.00) when compared with COVID-19 cases without CLD[48]. A United States-based multicenter study reported a mortality rate of 12% in COVID-19 patients with pre-existing liver disease compared with 4% in those without[49]. Li *et al*[5] found that patients with underlying chronic hepatitis B virus infection suffered from a higher rate of severe or critical COVID-19 illness than mild/moderate illness ($P < 0.0001$). However, it remains unclear whether COVID-19 infection causes an already susceptible liver to fail or is just a risk factor for fulminant hepatic failure[21]. Acute and chronic liver failure related to COVID-19 infection has been shown in patients with decompensated alcoholic and non-alcoholic liver cirrhosis[50,51]. Whether cases with cirrhosis and COVID-19 are at higher risk of decompensation or development of acute-on-chronic liver failure, as has been reported for influenza infection, remains undetermined [52]. Notably, existence of metabolic-associated fatty liver disease (MAFLD) was considered an independent factor for the severity of COVID-19 in a series of non-diabetic COVID-19 infected cases, indicating an injurious bidirectional relationship between liver disease and COVID-19 infection[53]. Obesity is an essential criterion in MAFLD[54], and it was reported that the severity of COVID-19 showed a six-fold increase in obese patients with MAFLD[28]. Furthermore, patients with MAFLD and a high fibrosis score were more liable to suffer from severe COVID-19 disease, regardless the existing metabolic abnormalities[55]. Large amounts of IL-6 are produced in patients with severe COVID-19, particularly those with obesity, and this is considered a primary factor in triggering a systemic inflammatory response and cytokine storm, as well as multiple organ dysfunctions[53,56]. Moreover, there is altered secretion of inflammatory lipid mediators and a reduction in adiponectin levels in obese patients with MAFLD[57]. In patients with underlying advanced CLD, SARS-CoV-2 infection could lead to hepatorenal syndrome and liver transplantation[50]. Liver transplant recipients and other immunosuppressed patients who have COVID-19 may have a longer duration of viral shedding than non-immunosuppressed patients[58]. Additionally, Center for Disease Control and Prevention considers patients on immunosuppressive therapy for autoimmune liver diseases (AILD) are at high risk for severe COVID-19 disease and have prolonged viral shedding[59]. Meanwhile, patients with well-controlled Wilson disease or with genetic hemochromatosis showed no increased risk of having COVID-19 infection[60]. On the other hand, children with CLD, including those with AILD and post liver transplant, do not have an increased risk for severe COVID-19 disease, with little or no liver dysfunction [61,62]. It was found that the risk of mortality in COVID-19 patients is associated with the severity of the underlying liver diseases[45,57]. A recent meta-analysis based on confounding cofactors-controlled data demonstrated that cirrhosis was an independent risk factor for prediction of mortality associated with SARS-CoV-2 infection[63].

SARS-COV-2 VACCINATION AND LIVER DISEASE

Patients with CLD can receive a COVID-19 vaccination, although the immunogenicity and effectiveness of these vaccines have not been fully evaluated in this group of patients. However, vaccination has been associated with a lower risk of COVID-19-related infection and mortality in patients with cirrhosis[4,64]. Following COVID-19 vaccination, immune-mediated liver injury (ILI) is not well-characterized. A recent meta-analysis of 23 patients (mean age, 55.3 years) showed jaundice as the most common symptom (78.3%). Peak bilirubin, ALT, and ALP levels were 10.8 (6.8–14.8) mg/dL, 1106.5 (757.0–1702.5) U/L, and 229 (174.6–259.6) U/L, respectively. Histological examination showed intense portal lymphoplasmacytic infiltrate with interface hepatitis. The mean duration between receiving the vaccine dose (either first or second) and subsequent development of liver injury was 17.3 (11.2–23.4) d. Steroids were used in 86.9% of cases, and complete response, recovery, and death were reported in 56.5%, 39.1%, and 4.3% of cases, respectively. A temporal course between vaccination and the onset of liver injury was reported. Shroff *et al*[65] noted that most cases of severe liver injury were described after SARS-CoV-2 mRNA vaccines. Most cases occurred following the first vaccination dose, and two developed ILI following the second dose. Interestingly, there was one case of ILI after both doses of vaccine. It is also notable that pre-existing comorbidities (69.6%) were common, including liver disease in 26.1% and thyroid disorders in 13% of patients[66].



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Figure 1 Summary of coronavirus disease 2019-induced liver dysfunction. MAFLD: Metabolic-associated fatty liver disease; AILD: Autoimmune liver diseases; COVID-19: Coronavirus disease 2019; ALT: Alanine transaminase; ULN: Upper limit of normal; AST: Aspartate transaminase; PICU: Pediatric intensive care; CLD: Chronic liver disease; HELLP: Hemolysis, elevated liver enzymes and low platelet; MISC: Multisystem inflammatory syndrome of children.

CONCLUSION

This editorial sheds light on liver involvement in COVID-19 patients with and without pre-existing liver injury. Further studies are necessary to elucidate the etiology and mechanism(s) of liver dysfunction associated with COVID-19 infection, particularly in patients aged less than 18 years. Liver function should be monitored carefully during COVID-19 infection.

FOOTNOTES

Author contributions: Said ZNA, El Habashy SA, and Zaky S contributed equally to this work; All authors have read and agreed to the published version of the manuscript.

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Country/Territory of origin: Egypt

ORCID number: Zeinab Nabil Ahmed Said 0000-0002-9788-9058; Safinaz Adel El Habashy 0000-0002-1115-4684; Samy Zaky 0000-0003-4123-9221.

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OPINION REVIEW

Tranexamic acid may be a useful pharmacotherapy for endoscopically resistant small bowel angiodysplasia

Shunji Fujimori

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Shunji Fujimori, Department of Gastroenterology, Chiba Hokusoh Hospital, Nippon Medical School, Chiba 270-1694, Japan

Corresponding author: Shunji Fujimori, AGAF, MD, PhD, Director, Department of Gastroenterology, Chiba Hokusoh Hospital, Nippon Medical School, 1715, Kamagari, Inzai-City, Chiba 270-1694, Japan. s-fujimori@nms.ac.jp

Abstract

Small bowel angiodysplasia (SBAD) is reported to account for nearly 50% of cases of small bowel bleeding. When SBAD occurs frequently, it is difficult to treat all the angiodysplasias endoscopically, and gastrointestinal bleeding often recurs. Hormone therapy, somatostatin analogs, thalidomide and vascular endothelial growth factor (VEGF)-neutralizing antibodies have been reported to reduce gastrointestinal angiodysplasia (GIAD) bleeding. However, there is no strong evidence to recommend them. Also, there are no guidelines for their use. Hereditary hemorrhagic telangiectasia (HHT) is a hereditary disease caused by abnormalities in VEGF, resulting in multiple GIADs. A treatment guideline has been created for GIAD in HHT, and the use of tranexamic acid, an antifibrinolytic agent, is the first recommendation pharmacotherapy for GIAD with gastrointestinal bleeding that is difficult to treat endoscopically. It has been reported that fibrinolysis is accelerated in GIAD patients who are not HHT, similar to HHT patients. The use of tranexamic acid for gastric antral vascular ectasia in GIAD has been reported to be useful. However, there are very few reports of its use for SBAD. There are concerns with tranexamic acid use regarding the development of thrombosis/embolism, but there are few reports of such side effects. Future clinical trials including tranexamic acid for SBAD are desired.

Key Words: Angiodysplasia; Intestine; Hereditary hemorrhagic telangiectasia; Tranexamic acid; Endoscopic treatment; Pharmacotherapy

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Core Tip: It is difficult to treat all multiple small bowel angiodysplasias (SBAD) endoscopically. Four main types of drugs, including somatostatin analogs, hormone therapy, thalidomide, and vascular endothelial growth factor-neutralizing antibodies, have been reported for use in gastrointestinal angiodysplasias (GIAD). However, there is no recommended pharmacotherapy for SBAD. Tranexamic acid is recommended for patients with GIAD in hereditary hemorrhagic telangiectasia who are difficult to treat endoscopically. Investigation of the use of tranexamic acid for SBAD is desired.

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INTRODUCTION

Gastrointestinal angiodysplasia (GIAD) is a benign vascular malformation of the GI tract and is a frequent source of GI bleeding. AD has been reported under various names, such as vascularectasia, angioectasa, and angiectasia, but since all of these generally present with the same lesions, they are collectively referred to here as AD. GIAD patients include patients with isolated AD in the stomach, small bowel, and large bowel; gastric antral vascular ectasia (GAVE) secondary to cirrhosis; and Heyde's syndrome secondary to aortic stenosis. GIAD causes 10% of all GI bleeding cases and 50% of small bowel bleeding cases[1]. GIAD has also been reported to cause approximately 4%-7% of upper nonvariceal bleeding, 30%-40% of occult small bowel bleeding, and 3%-40% of colonic bleeding episodes [2].

GIAD diagnosed as a source of GI bleeding often causes rebleeding even after endoscopic treatment, and subsequent treatment is difficult in these cases. After hospitalization and treatment in patients diagnosed with GIAD as the source of GI bleeding, the rate of rebleeding reaches 20% 30 d after discharge, and medical expenses are high[3]. In addition, a systematic review showed that 42.7% (209/490) of small bowel AD (SBAD) patients had rebleeding even after endoscopic treatment, and subsequent treatment is difficult in cases of rebleeding[4].

A major reason for rebleeding is that ADs tend to occur frequently. Even gastric ADs such as GAVE occur so frequently that endoscopic treatment is difficult. In addition, it has been reported that 2 or more ADs in the small bowel were observed in more than 63% of patients[5]. It is difficult to perform enteroscopy frequently for multiple SBADs, and it is difficult to find all the ADs in the long length of the small bowel. In other words, even if one lesion is treated, the other lesions bleed. Some types of pharmacotherapies are desirable for patients with SBAD whose overt bleeding or anemia progresses even after multiple endoscopic treatments. There are no established pharmacological treatments for SBAD. However, several pharmacological treatments for GIAD have been proposed, such as somatostatin analogs, hormone therapy, thalidomide and angiogenesis inhibitors.

Hereditary hemorrhagic telangiectasia (HHT) is a congenital disease in which AD occurs frequently in various organs. In HHT, GIADs occur so frequently that endoscopic treatment is difficult. Therefore, the pharmacotherapy of HHT has been studied for a long time, many reports have been published, and guidelines have been established. In this paper, the GI bleeding section of the HHT guideline is outlined, the pharmacotherapy options for GIAD that have been performed thus far are introduced, and finally, the treatment policy for SBAD is considered based on the HHT guideline.

GUIDELINE FOR HHT

HHT is a multiorgan disease in which AD occurs frequently in the skin, mucous membranes, and GI tract, causing recurrent bleeding and arteriovenous malformations (AVMs) in the brain, spinal cord, lungs, and liver[6,7]. HHT is inherited in an autosomal dominant manner and is caused by abnormalities in vascular endothelial growth factor (VEGF)[8]. The gene associated with HHT type 1 is a transforming growth factor-beta binding protein of endothelial cells[9]. HHT types 1 and 2 arise from mutations in endoglin and activin receptor-like kinase 1, respectively[10].

Epistaxis is a serious problem in HHT patients, but GI bleeding due to multiple GIADs is also a major problem. HHT has international guidelines, updated in 2020[11]. The guidelines recommend endoscopic treatment for GIAD in patients with HHT first. Second, antifibrinolytic therapy is encouraged as pharmacotherapy for endoscopic treatment resistant cases. However, the degree of encouragement is weak. This guideline is due to a recent report that showed that the antifibrinolytic agent tranexamic acid reduced the need for endoscopic management in patients with HHT[12]. The latest report summarizing the main points of the guideline recommends using tranexamic acid as the first step after performing

argon plasma coagulation of actively bleeding lesions at the initial diagnosis. Systemic antiangiogenic agents are recommended as a next step if no improvement is seen with administration of tranexamic acid[13]. The guideline committee states that, where possible, the use of dual antiplatelet therapy and/or a combination of antiplatelet therapy and anticoagulation should be avoided in patients with HHT. If anticoagulation is not tolerated in HHT patients with atrial fibrillation, discontinuation of anticoagulation with alternative approaches, such as left atrial appendage closure, is recommended[14]. Since the bleeding in HHT patients tends to be serious, the use of anticoagulant therapy and antiplatelet therapy is recommended with the minimum amount that is necessary.

Patients with multiple SBADs often have fewer ADs than patients with HHT. However, SBAD is a similar pathology, and HHT is a reference for SBAD treatment. There are no trials of tranexamic acid in the pharmacological treatment of SBAD in non-HHT patients, nor does tranexamic acid appear in various reviews as a treatment for SBAD. The next section considers the drug treatment of GIAD, especially SBAD, with reference to HHT.

PHARMACOLOGICAL TREATMENT OF GIAD

According to an analysis of the MEDLINE, Cochrane, Scopus and Embase databases, there are four main pharmacological therapies for GIAD that have been reported: Somatostatin analogs, hormone therapy, thalidomide and angiogenesis inhibitors[15]. These drugs are discussed first, followed by a discussion of tranexamic acid, which is used in the treatment of HHT.

Hormone therapy

Studies of oral contraceptives have shown that there is a risk of thrombosis with hormonal therapy with estrogens and progestogens[16]. It has been reported that this hormone therapy affects blood clotting ability and shortens bleeding time[17], which leads to its use as a hemostatic agent. Activated protein C resistance was reported to be involved in this coagulation enhancement[18]. On the other hand, postmenopausal hormonal therapy has been reported to increase plasma fibrinolytic activity, plasma levels of D-dimer and tissue plasminogen activator activity[19]. Thus, the therapy does not appear to increase coagulability in postmenopausal women. The increase in plasma fibrinolytic activity due to postmenopausal hormone therapy may be considered a secondary phenomenon of hypercoagulability. Fundamentally, hormone therapy for GIAD is considered to be an attempt to utilize the enhancement of blood coagulability for hemostasis against GI bleeding.

In a double-blind, placebo-controlled, crossover trial using estrogens and progestogens in 10 patients with GIAD requiring frequent blood transfusions, it was reported that hormone therapy reduced the amount of transfusion[20]. Various studies have originated from this Lancet report in 1990. In a prospective observational study with an average follow-up period of 535 d in 43 patients with obscure GI bleeding, all 38 who received concomitant hormonal therapy were bleeding-free. In contrast, all 5 patients treated with estrogen alone had episodes of rebleeding[21]. However, a double-blind randomized control trial (RCT) was conducted in 72 noncirrhotic patients with hemorrhages of GIAD-probable origin; rebleeding occurred in 46% of the 35 patients; and no significant difference was observed[22]. As a result of this study, the number of reports on hormone therapy has decreased, but reports on treatment using hormone therapy have been published recently. For example, when comparing 6 mo before and after hormone therapy for GIAD, anemia improved, and the number of blood transfusions decreased[23]. Hormone therapy has side effects peculiar to hormone drugs, but it is generally safe and has the advantage of being inexpensive. For these reasons, the search for a hormone therapy treatment for difficult-to-control GI bleeding continues.

Somatostatin analogs

Since the 1990s, attempts have been made to use the somatostatin analogs octreotide and lanreotide to treat hemorrhagic GIAD. Somatostatin analogs for GIAD are thought to inhibit angiogenic promoters by affecting chemical factors such as VEGF, basic fibroblast growth factor, and insulin-like growth factor 1 and to stimulate relaxation of intestinal smooth muscle, relieve chronic submucosal vein occlusion, and reduce the intravascular pressure on the arterial side[24]. There are many reports showing that somatostatin analogs are effective in patients with GIAD, but most have a small number of patients and are retrospective studies[1]. The usefulness of somatostatin analogs was reported in an RCT targeting 70 patients in which the actuarial probability of remaining free of rebleeding at 1 and 2 years of follow-up was 77% and 68%, respectively, in the octreotide group and 55% and 36%, respectively, in the placebo group ($P = 0.030$)[25].

Recently, a systematic review of 212 patients from 11 studies investigating the usefulness of somatostatin analogs for patients with GIAD, with a median duration of treatment of 12 mo, was reported. Somatostatin analogs were reported to reduce the number of red blood cell transfusions by an incidence rate ratio of 0.18 ($P < 0.0001$). The most common side effects of somatostatin reported in this study were loose stools (3%), cholelithiasis (2%), flatulence (2%), and administration site erythema (2%)[26]. Another systematic review and meta-analysis suggested that somatostatin analogs are more useful

in patients with GIAD than hormone therapy[27]. Based on the above, somatostatin analogs are thought to be useful for the treatment of patients with GIAD. However, since most somatostatin analogs are a daily subcutaneous injection and are expensive, there is a high possibility that the number of patients that can be treated with them are small.

Thalidomide

Thalidomide was reported to inhibit angiogenesis by suppressing VEGF[28]. Recently, it was reported that EGF-like domain multiple 6 (EGFL6) is overexpressed in patients with SBAD, and *in vitro* and *in vivo* assays reveal that thalidomide can act as an anti-angiogenic agent through the regulation of EGFL6 in a proteasome-dependent manner[29].

One RCT showing the efficacy of thalidomide for patients with GIAD has been published. The study randomized 55 patients with GIAD to receive either thalidomide 100 mg (n = 28) or iron 400 mg (n = 27, controls) daily for 4 mo. The treatment was considered to be effective when patients showed a 50% or greater reduction in bleeding episodes (fecal occult blood) in the first year of follow-up; the response rates in the thalidomide group and the control group were 71.4% and 3.7%, respectively (P < 0.001)[30].

Thalidomide is also considered useful in patients with HHT[31]. Therefore, thalidomide is listed as one of the systemic antiangiogenic therapies in the HHT guidelines[11]. However, thalidomide is highly teratogenic, and since it was developed as a sleeping drug, it has been shown to cause neurological symptoms such as somnolence in a drug-dependent manner. These issues limit its use.

VEGF-neutralizing antibodies

As previously mentioned, HHT is caused by abnormalities in VEGF. First, a VEGF-neutralizing antibody was reported to prevent cutaneous AVM formation and ameliorate the internal bleeding in Alk1-deficient adult HHT model mice[32]. In animal experiments, sorafenib and a pazopanib analog were reported to be effective against a mouse model of HHT, and there have been many case reports of the effectiveness of bevacizumab for HHT[33-39]. However, since these are all case reports, generally, VEGF-neutralizing antibodies cannot be judged to be effective, and further investigation is needed.

On the other hand, patients with GIAD did not show any abnormalities in their VEGF levels. In patients with GIAD, angiopoietin-2 was increased, but VEGF did not increase[40]. Similarly, when the serum from patients with SBAD, portal hypertensive gastropathy, and GAVE was compared with the serum from nonbleeding, nonanemic control patients, angiopoietin-2 was increased, but there was no difference in the blood VEGF concentration[41]. Although the relationship between GIAD and VEGF is not clear, there are reports that VEGF-neutralizing antibodies are effective in patients with GIAD. A study reported that bevacizumab was effective for treating GIAD in two patients with Heyde's syndrome[42]. There is one case report involving Heyde's syndrome[43] and another case report showing that bevacizumab is useful for patients with GIAD[44]. Since the number of reports is still small, VEGF-neutralizing antibodies cannot be judged to be effective, and further examination is necessary. Even if effective, VEGF-neutralizing antibody preparations are extremely expensive and difficult to use in many patients.

Tranexamic acid

Plasma hyperfibrinolysis has been reported to occur in both patients with HHT[45] and patients with hemorrhagic GIAD[46]. The latter report suggests that intrinsic ischemia may be the cause of GIAD, which may result in increased fibrinolytic activity in patients with GIAD. This plasma hyperfibrinolysis may promote GI bleeding in patients with GIAD. Tranexamic acid has been widely used as a hemostatic agent for many bleeding disorders by utilizing its anti-hyperfibrinolysis action. Tranexamic acid is a synthetic derivative of the amino acid lysine and exerts antifibrinolytic effects through reversible blockade of the lysine binding site on the plasminogen molecule[47].

A comparative study published in 1973 showed that tranexamic acid is effective in stopping bleeding in the upper GI tract[48]. Gastric juice has been reported to induce marked fibrinolysis, and tranexamic acid was expected to stop bleeding, especially in gastric lesions[49]. In a meta-analysis, tranexamic acid was shown to reduce mortality in patients with upper GI bleeding by 5%-54% and by 40% compared to placebo and is expected to be effective in a variety of nongastrointestinal bleeding disorders[47]. Tranexamic acid was also used for GAVE in patients with cirrhosis that was unresponsive to propranolol therapy and transjugular intrahepatic portosystemic shunt and was reported to reduce bleeding by 20%-30% and the need for surgery by 30%-40%[50-52].

As mentioned above, although the recommendations for it are weak, tranexamic acid is considered to be the first-choice drug for GI bleeding in patients with HHT that is difficult to treat endoscopically. For GIAD, it was reported for the first time in 1998 that tranexamic acid is effective for treating chronic bleeding from colonic AD in dialysis patients[53]. Jejunal AD with persistent bleeding is called Bernard-Soulier, and it was reported in 2013 that tranexamic acid is an effective treatment[54]. Tranexamic acid was reported to be very effective in a patient with multiple duodenal and jejunal ADs who had a medical history of ineffective hormone therapy; discontinued thalidomide treatment due to side effects such as nausea, dizziness, and severe fatigue; and discontinued octreotide treatment after one dose due to a hypoglycemic episode[55]. However, there are still only a few reports of the use of tranexamic acid

Table 1 Mechanisms of drugs used to treat gastrointestinal angiodysplasia and the main studies

Drug/therapy	Mechanism	Leading clinical trial	No. of cases	Result	Ref.
Hormone therapy	Enhanced coagulability				
		Double-blind, placebo-controlled, crossover trial for GIAD	10	Effective	[20]
		Prospective observational study for OGIB	43	Effective	[21]
		Double-blind RCT for GIAD	72	Not effective	[22]
		Systematic review for GIAD	63	Not effective	[27]
		Comparing before and after therapy for GIAD	12	Effective	[23]
Somatostatin analogs	Inhibition of angiogenic promoters				
	Relaxation of intestinal smooth muscle	RCT for gastrointestinal bleeding due to GIAD	70	Effective	[25]
		Systematic review for GIAD	72	Effective	[27]
		Systematic review for GIAD	212	Effective	[26]
Thalidomide	Anti-angiogenic agent				
		RCT for GIAD	55	Effective	[30]
VEGF-neutralizing antibodies	Angiogenesis inhibition				
		Only case reports			
Tranexamic acid	Antifibrinolytic effects				
		Observational study for GAVE	8	Effective	[51]
		Retrospective study for HHT	42	Effective	[12]

OGIB: Obscure gastrointestinal bleeding; GAVE: Gastric antral vascular ectasia; HHT: Hereditary hemorrhagic telangiectasia; GIAD: Gastrointestinal angiodysplasia; RCT: Randomized control trial; VEGF: Vascular endothelial growth factor.

for patients with SBAD.

Since tranexamic acid is an antifibrinolytic agent, there are concerns about thrombosis and embolism, and the following reports have been published. First, there are reports of ischemic episodes and pulmonary embolisms with the use of tranexamic acid[52]. In addition, there is a case report in which tranexamic acid was used to treat SBAD in dialysis patients for whom endoscopic treatment was difficult, and thrombosis of the arteriovenous fistula occurred[56]. In contrast, a meta-analysis of 4747 patients undergoing cesarean section or vaginal delivery found no association between tranexamic acid and deep vein thrombosis[57]. Additionally, in an RCT conducted in patients undergoing bilateral total knee arthroplasty, adverse effects such as deep vein thrombosis and pulmonary embolism were not significantly different between 245 patients who received tranexamic acid and 271 who did not[58]. Furthermore, a review of short-term tranexamic acid use in postdental surgery patients on anticoagulant therapy found no complications, such as thrombosis, in 125 tranexamic acid-treated patients[59]. Based on these reports, tranexamic acid appears to be relatively safe for short-term use. However, there have been no reports on the long-term use of tranexamic acid, especially during anticoagulant or antiplatelet therapy for myocardial infarction or cerebral infarction, and caution should be exercised when using tranexamic acid in patients with these high-risk diseases. Although there are concerns about the risks described above, tranexamic acid is an inexpensive drug that can be expected to reduce the amount of GI bleeding in patients with SBAD who are difficult to treat by endoscopy. Future reports are needed.

CONCLUSION

Table 1 summarizes the drugs currently being studied for use in patients with GIAD, including SBAD. There are several reports that hormone therapy, somatostatin analogs, thalidomide and VEGF-neutralizing antibodies are useful for SBAD for which endoscopic treatment is difficult. Hormone therapy is a good choice considering its less side effects and costs, but there are negative reports. Due to the small number of reports, it is not possible to decide which drug to strongly recommend. Tranexamic

acid has been adopted as a first-line pharmacological treatment in the guidelines for GIAD in HHT patients who are difficult to treat endoscopically. It is also effective for GAVE in non-HHT patients. Although there are concerns about the risk of thrombosis and embolism, tranexamic acid is expected to reduce the amount of GI bleeding in patients with SBAD in whom endoscopic treatment is difficult. Future reports are expected, as tranexamic acid could be a first-line drug for patients with SBAD.

FOOTNOTES

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Country/Territory of origin: Japan

ORCID number: Shunji Fujimori 0000-0002-6214-2595.

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P-Editor: Wang JJ

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REVIEW

Are we ready for telemonitoring inflammatory bowel disease? A review of advances, enablers, and barriers

Javier Del Hoyo, Mónica Millán, Alejandro Garrido-Marín, Mariam Aguas

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Javier Del Hoyo, Alejandro Garrido-Marín, Mariam Aguas, Department of Gastroenterology, La Fe University and Polytechnic Hospital, Valencia 46026, Spain

Mónica Millán, Department of Surgery, La Fe University and Polytechnic Hospital, Valencia 46026, Spain

Mariam Aguas, Health Research Institute La Fe, La Fe University and Polytechnic Hospital, Valencia 46026, Spain

Corresponding author: Mónica Millán, MD, PhD, Doctor, Staff Physician, Department of Surgery, La Fe University and Polytechnic Hospital, Av. Fernando Abril Martorell, Valencia 46026, Spain. monicamillan72@gmail.com

Abstract

This review summarizes the evidence about telemonitoring in patients with inflammatory bowel disease (IBD). To give an overview of the advances performed, as well as the enablers and barriers which favoured/hindered telemonitoring implementation. We performed a literature search in PubMed, EMBASE, MEDLINE, Cochrane Database, Web of Science and Conference Proceedings. Titles and abstracts published up to September 2022 were screened for a set of inclusion criteria: telemonitoring intervention, IBD as the main disease, and a primary study performed. Ninety-seven reports were selected for full review. Finally, 20 were included for data extraction and critical appraisal. Most studies used telemonitoring combined with tele-education, and programs evolved from home telemanagement systems towards web portals through mHealth applications. Web systems demonstrated patients' acceptance, improvement in quality of life, disease activity and knowledge, with a good cost-effectiveness profile in the short-term. Initially, telemonitoring was almost restricted to ulcerative colitis, but new patient reported outcome measures, home-based tests and mobile devices favoured its expansion to different patients´ categories. However, technological and knowledge advances led to legal, ethical, economical and logistic issues. Standardization of remote healthcare is necessary, to improve the interoperability of systems as well as to address liability concerns and users´ preferences. Telemonitoring IBD is well accepted and improves clinical outcomes at a lower cost in the short-term. Funders, policymakers, providers, and patients need to align their interests to overcome the emerging barriers for its full implementation.

Key Words: Inflammatory bowel disease; Telemedicine; Telemonitoring; Information and

communication technology; Crohn´s disease; Ulcerative colitis

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Core Tip: In this review we focus on the advances performed in telemonitoring of patients with inflammatory bowel disease, taking into consideration the elements which enabled its use and how technological achievements led to other barriers for its full implementation. We detail the impact of telemonitoring on health outcomes and its cost-effectiveness. We also describe the advances on new patient-reported outcome measures, home-based tests and wearables which improve the ability to manage new patients´ profiles remotely. However, during the pandemic, e-mail and telephone still represented the main resources used. Then, we describe the emerging barriers which explained the limited application of mature telemonitoring programs.

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INTRODUCTION

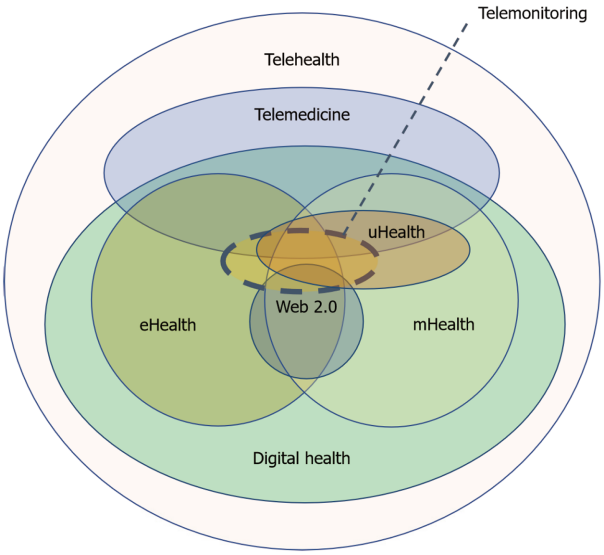
Inflammatory bowel disease (IBD) is a group of disorders characterized by the chronic and recurrent inflammation of different segments of the gastrointestinal tract, which usually associates extraintestinal manifestations and complications due to sustained activity. Unlike other chronic pathologies, IBD mainly affects young individuals in their optimal period of personal and professional development. As such, IBD is related to high levels of school absenteeism and work disability[1], interference with social activities, and impaired health-related quality of life (QoL)[2]. Therefore, IBD has a significant medical, social, and financial impact, further increased by the global increase in its incidence and prevalence in recent years[3].

It is suggested that the "treat-to-target" strategy leads to better outcomes[4]. However, in the conventional management of IBD, scheduled outpatient visits show difficulties to address the disease evolution in each patient, with frequent discrepancies between medical practice and guideline recommendations. Furthermore, patients have little involvement in decision-making, and nearly 50% of adults[5] and an even higher percentage of adolescents with IBD[6,7] are nonadherent to treatment. All these factors prevent the effectiveness of traditional interventions in disease control and increase health expenses[8], especially considering that patients with IBD use health care resources more often than patients with other conditions[9].

Nowadays, health systems are facing financial problems, and telemedicine has been proposed as an alternative to provide an efficient and equitable use of health resources. Information and communication technologies (ICTs) have the potential advantages of providing better communication between healthcare providers and patients, as well as educational resources adapted to patients´ needs. On the one hand, communication improvements could overcome limitations of health access in remote areas, also developing telementoring systems and contact between different specialists in centres where multidisciplinary teams are not available. On the other hand, educational elements could favour patients' empowerment and treatment optimization throughout the disease course[10,11], also addressing behavioural and psychological factors related to nonadherence[5].

Telemedicine has been successfully used in other chronic diseases such as congestive heart failure[12,13], diabetes mellitus[14,15] or chronic obstructive pulmonary disease[16,17] and showed excellent acceptance by patients, improvement in health related QoL and a reduction in hospitalizations[12,16,18]. Owing to these positive results, telemedicine systems have been evaluated in patients with IBD, especially in mild to moderate ulcerative colitis (UC)[19,20]. Telemedicine in IBD started with the adaptation of telemonitoring programs previously used in other chronic pathologies[21], but these were subsequently replaced by web and m-health systems, which represented more attractive options to maintain patients adherence to remote follow-up.

Telemonitoring is the main form of telemedicine in IBD. It is based on the provision of health services at a distance, related to diagnosis, treatment, follow-up or education. It is characterized by the structured and continuous monitoring of clinical data that is self-reported by patients in their usual environment, and then sent to health providers. The objective is the early detection and intervention on complications related to the disease itself or its treatment. It usually includes tele-education interventions and shares many features with other domains of use of ICTs in the health-care setting (Figure 1). Web telemonitoring in IBD is safe and reduces the duration of disease flares[22]. Moreover,



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Figure 1 Telemonitoring in relation to other domains associated with the use of information and communication technologies in the health context.

patients´ empowerment has been related to a reduction in outpatient visits and hospitalizations[23-26], which represent potential cost savings[22,24,27].

The development of more sophisticated telemonitoring programs and point of care (PoC) testing during recent years provided additional value to remote follow-up in the IBD context, improving the ability to cover different patients' profiles more objectively. These advances gained special interest after the advent of coronavirus disease 2019 (COVID-19) outbreak, as distance management offered new elements to overcome healthcare challenges posed during the pandemic[28]. However, telemedicine was represented mainly by telephone and e-mail, previously available in many centres[29,30], but the development of mature telemedicine programs integrated with electronic health records were still the exception, due to a series of remaining barriers.

In this review we focus on the advances performed in telemonitoring of patients with IBD, taking into consideration the elements which enabled its use and how technological achievements led to other barriers for its full implementation. The search strategy is detailed in the Supplementary material.

TELEMEDICINE IN CHRONIC DISEASES

There is a wide heterogeneity of telemedicine programs considering the different types of technological resources used, the different diseases and populations in which they are applied, and the objectives pursued with their use. The variability in the quality and design of the published studies (randomized clinical trials, before-and-after studies, qualitative studies, *etc*.) can partially explain the variable results obtained. Furthermore, in some studies these systems are part of wider interventions, rendering the comparability between programs even more difficult. These factors limit the quality of evidence regarding the efficacy of telemedicine to improve outcomes in chronic diseases.

Despite this, ICTs have been used in a wide range of pathologies, with improvement of patients´ empowerment and with good acceptance[31]. With the aim of giving response to the rise in chronic diseases and multimorbidity worldwide, different projects in Europe have studied the use of telehealth, mainly in diabetes mellitus, cardiovascular diseases, depression, and chronic obstructive pulmonary disease (COPD).

There is moderate evidence about the efficacy of telehealth systems in the improvement of glycaemic control, mainly in terms of HbA1c in patients with type 2 diabetes mellitus. Active telemonitoring including providers´ feedback, as well as tele-education have shown positive results compared with usual practice[14]. In fact, the highest impact was seen in the combination of telemonitoring and tele-education for both patients and providers, allowing for shared decision-making[15].

In patients with heart failure, telemonitoring has been shown to reduce global mortality and hospitalizations compared to usual care[12]. Many telemonitoring systems were part of multidisciplinary programs managed by specialized nurses and incorporated tele-education and action plans before hospital discharge[13]. Interactive monitoring with healthcare providers has also shown to improve blood pressure in hypertensive patients, weight control and lipidic profile[32]. Video consultation and biosensors are especially useful in cardiovascular diseases, with reduced costs compared with other

pathologies[33].

Most digital resources used in the mental health context refer to the application of cognitive behavioural therapy (CBT) in patients with depression. Both traditional and e-Health CBT are effective [34], but its use with telemedicine programs could offer additional advantages, such as better accessibility. However, many studies with psychotherapies show a high rate of nonadherence to follow-up [35]. Similarly, in patients with IBD one clinical trial showed a significant improvement in QoL after 12 wk of self-administered computerized CBT, but this outcome was not maintained at 6 mo, with a high rate of dropouts[36].

In patients with asthma, the use of multiplatform programs combining tele-education, telemonitoring and individualized action plans reduced hospitalizations compared with traditional care, mainly in more severe patients[18]. In patients with COPD, the use of telemedicine also reduces hospitalizations, but without an improvement of global mortality[16]. With the development of mHealth, the use of SMS combined with telephone support is associated with an improvement in respiratory function and QoL in patients with asthma[32], but telemonitoring in COPD has not demonstrated any improvement in these outcomes[17,37]. Telemonitoring of patients with COPD is more expensive due to associated multimorbidity[33].

The use of telemedicine in digestive diseases is more limited, and most studies focused on IBD and irritable bowel syndrome. Unlike other chronic diseases such as diabetes mellitus, IBD implies the consideration of many clinical, biological, endoscopic, and even histologic variables to reach disease control. However, early detection of complications usually requires invasive tests in IBD. The absence of validated tools with adequate cost and accuracy to measure disease activity at a distance have represented an important limitation.

Most studies about telemedicine in IBD have emerged in the last decade. The development of mHealth, the validation of patient reported outcome measures (PROMs), PoC and home-based tests to measure fecal calprotectin (FC) near the patient improved the ability to evaluate more types of patients with IBD at a distance, even in more complex cases.

PROGRAMS FOR TELEMONITORING IBD

The increase in the capacity of data transmission and storage, as well as the evolution of wireless communications provided many resources that are easy to use and adaptable to IBD telemonitoring.

Initially, telemonitoring systems for IBD allowed communication between health centres and patient´s home using computers. Afterwards, the development of web-based systems permitted easy-to-use and cheaper telemonitoring programs. In the last years, mobile devices (Smartphone, Tablet, *etc.*) made it possible to establish the communication process with the patient during his/her daily activities. Furthermore, in other settings (such as cardiovascular diseases) the transmission of continuous physiological data through biotelemetry has evolved with the incorporation of wearables.

In the IBD setting, telemonitoring is a safe, acceptable, and effective option to improve clinical outcomes[38,39], but the results of studies are still variable. In this context, telemonitoring has mainly used programs requiring home installation or web-systems, although e-mail and telephone have supported some of these programs. The main telemonitoring platforms used in IBD are summarized in Table 1.

Home telemanagement systems

The Cross group was the first to apply ICTs in adult patients with IBD, mainly with UC. This research team developed a remote-control system (home automated telemanagement system: HAT system) made up of 3 stations, adapted from a program previously used in self-management of patients with asthma [40]. The Home Unit was made up of a portable computer that collected patients' information (symptoms, adverse effects, medication, *etc.*), and these data were then sent to a decision-support server connected to a provider's PC[21]. The computer created alerts if the values collected in a web portal surpassed pre-established thresholds. Moreover, the HAT system incorporated educative elements.

Different exploratory studies showed good acceptance with the use of this system. In 2 studies with 10 and 23 patients with IBD, all of them considered that the HAT system was simple and increased patients´ knowledge[21,41]. To confirm the acceptability and adherence to follow-up with this program, the authors performed a subsequent study with 25 patients followed-up over 6 mo. Adherence to the weekly questionnaire was 91% and 86% had a prescribed medication adherence over 80%. This good adherence corresponded to a trend towards improvement in disease activity and QoL levels, together with a statistically significant improvement in understanding the disease (P = 0.0015). These good results led to the hypothesis that the HAT system could be feasible for telemonitoring patients with IBD.

Subsequently, the same group designed a randomized clinical trial including 47 patients with mild to moderate UC. Twenty-five patients were controlled with the HAT system and 22 followed usual in-person visits together with educational support and individualized action plans to make groups more comparable[42]. The groups had similar baseline characteristics, except for the use of immunosuppressants in 56% of the study group and 27% of the control group (P = 0.05), which would indicate a

Table 1 Studies of Telemonitoring in inflammatory bowel disease

Ref.	Disease	Type of study	*n*	Application	Outcomes
Cross *et al*[21]	IBD	Noncontrolled, clinical trial	10	Telemonitoring, home unit-server PC provider	Feasible method Excellent patient acceptance
Cross *et al*[113]	IBD	Noncontrolled clinical trial	25	Telemonitoring, home unit-server PC provider	Feasible method Excellent patient acceptance Improvement in QoL, disease activity, and disease knowledge
Cross *et al*[42]	UC	Controlled randomized clinical trial	47	Telemonitoring, home unit-server PC provider	Feasible method Excellent patient acceptance Improvement in QoL
Elkjaer *et al*[115]	UC	Validation study in 2 groups	21	Telemonitoring through the web	Feasible method Excellent patient acceptance
Elkjaer *et al*[22]	UC	Controlled randomized clinical trial	333	Telemonitoring through the web	Feasible method Excellent patient acceptance Improvement in QoL, disease knowledge, and adherence
Pedersen *et al*[10]	CD	Pilot study, controlled	27	Telemonitoring through the web	Feasible and safe method for individualized scheduling of maintenance IFX treatment
Pedersen *et al*[11]	UC	Prospective noncontrolled study	95	Telemonitoring through the web	Feasible and improve adherence to therapy
Torrejón *et al*[56]	IBD	Descriptive, observational, retrospective	1784	Telecare through e-mail, phone calls, fax	Increased telematic contacts and decreased in-person care
Johnson *et al*[27]	IBD	Telemonitoring project	420	A web-guided programme	Effective, safe and cost savings
De Jong *et al*[24]	IBD	Controlled randomized clinical trial	909	Telemonitoring through the web (mHealth)	Reduced outpatient visits and hospitalizations
Carlsen *et al*[43]	IBD	Controlled randomized clinical trial	53	Telemonitoring through the web (mHealth)	Reduced outpatient visits No differences in disease activity, QoL or adherence compared with standard care
Walsh *et al*[112]	UC	Pilot study, non controlled	66	Telemonitoring through the web (mHealth)	Feasible and usable to measure disease activity, QoL and medication use
Del Hoyo *et al*[52]	IBD	Controlled randomized clinical trial	63	Telemonitoring through the web	Higher improvement in disease activity compared to usual care Similar improvement in QoL, social activities and satisfaction between groups
Cross *et al*[25]	IBD	Controlled randomized clinical trial	348	Telemonitoring through the web (mHealth)	Improvement in disease activity and QoL, although not superior to usual care Decrease in hospitalizations and increase in distance contacts
Bilgrami *et al*[48]	IBD	Controlled randomized clinical trial	222	Telemonitoring through the web (mHealth)	No differences in self-efficacy or patient activation compared with standard care
Schliep *et al*[47]	IBD	Controlled randomized clinical trial	217	Telemonitoring through the web (mHealth)	No significant improvement in depressive symptoms or QoL compared with standard care
Heida *et al*[45]	IBD	Controlled randomized clinical trial	170	Telemonitoring through the web, e-mail and telephone	Similar improvement in QoL compared to conventional care Reduction in outpatient visits and societal costs Satisfaction
Linn *et al*[46]	IBD	Controlled randomized clinical trial	160	Telemonitoring through the web or SMS combined with tailored counselling	Improved self-efficacy Satisfaction

Bonnaud *et al* [51]	IBD	Controlled randomized clinical trial	54	Telemonitoring through the web (mHealth)	Significant improvement in QoL A trend to reduce outpatient visits Satisfaction
McCombie *et al*[50]	IBD	Controlled randomized clinical trial	100	Telemonitoring through the web (mHealth) and home-based FC	Non-inferiority of QoL and symptoms Reduced outpatient visits

IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease; QoL: Quality of life

higher level of disease activity in the experimental group. There were no statistically significant differences for improvement of disease activity, treatment adherence, and quality-of-life values between both groups at 12 mo. These results could be related to the small sample size as well as a higher dropout rate in the intervention group, possibly due to the platform design, which required installation and eventual repairs at home.

To avoid these problems, Cross and cols developed web telemonitoring using mobile devices[25].

Web-based systems

In the last decade, telemonitoring systems have progressively evolved with web programs and mHealth solutions. Web applications are less expensive, safe, and feasible in the management of IBD not only in adults but also in adolescents[43-45], and they are associated with a reduction in outpatient visits and hospitalizations[22,24,27,43,45].

A Danish group developed telemonitoring through the web under the concept of "Constant-care". The system was developed through the web http://www.constant-care.dk, which also incorporated an educational centre. These investigators designed a randomized controlled trial with 333 UC patients treated with 5-aminosalicylates (5-ASA) from different hospitals in Denmark and Ireland[22]. The intervention group introduced clinical data and analytic results in the web to guide changes in the follow-up schedule and treatment. This intervention was compared to usual care.

After 12 mo of follow-up, in both the Danish and Irish population 88% of patients showed good acceptance with the web telemonitoring. There was a statistically significant improvement in adherence to treatment after 4 wk and a lower duration of disease flares. This was related to the use of high doses of 5-ASA in 100% of patients from the intervention group in Denmark, who also had improved QoL and disease knowledge. However, these outcomes were not reproduced in the Irish population. Moreover, in the Danish population telemonitoring reduced outpatient and emergency department visits, which led to direct cost-savings of 189 euros per patient-year, but also to an increase of e-mails and telephone contacts.

The use of this web-system in paediatric patients also reduced outpatient visits and school absenteeism, without differences in disease activity, QoL and adherence to treatment compared to the control group[43]. In another study developed in the University of California with a telemonitoring program combined with tele-education, patients followed remotely used less corticosteroids and suffered less hospitalizations and emergency department visits, with cost reductions of 16%[23]. In short, these studies show that web-telemonitoring is feasible, safe and could reduce health costs, although there are reproducibility differences depending on the population in which telemonitoring is applied[19].

Moreover, web-systems have been used to individualize the treatment according to the disease course. In a prospective study with 95 patients with mild to moderate UC, web control allowed the adjustment of 5-ASA doses and improved adherence. This was related with a significant improvement in clinical activity and FC values after 3 mo of follow-up[11]. Telemonitoring has even been used to individualize the treatment schedule with infliximab. After 1 year of follow-up, there were no significant changes in disease activity and QoL, although there was an estimated cost-saving of 699 euros/patient, compared with a historic control group[10].

In the same line and to avoid problems generated with the HAT system in the pioneering studies, the Cross group developed a web system for the management of patients with IBD (TELE-IBD) through text messages. In a randomized clinical trial with 3 parallel groups (TELE-IBD weekly, TELE-IBD every other week and control group) in 3 reference centres for IBD, they included 348 patients who had at least one disease flare in the last 2 years. Seventy-five percent completed the study, with an improvement in disease activity and QoL in the 3 groups, but without a higher improvement in these outcomes, depressive symptoms, or self-efficacy in the web control group, although in another study self-efficacy improved when tailored counselling was associated[46]. Moreover, telemonitoring was associated with a change in the profile of health expenses. Less hospitalizations were seen in the telemonitoring group but with higher use of non-invasive tests and telephone or e-mail[25,47,48].

The largest clinical trial with a telemonitoring program to date was performed with the Dutch web myIBDcoach (http://www.mijnibdcoach.nl). This web allows distance monitoring of disease activity, treatment adherence and side effects, as well as nutritional status, smoking habits, QoL, fatigue, stress,

anxiety and depression. As other platforms, it provides educational elements to improve empowerment. Patients showed good acceptance with its use in a pilot study[49]. In a clinical trial including 909 patients with different disease characteristics, the use of this system reduced outpatient visits and hospitalizations compared to standard care after 12 mo of follow-up[24]. Similarly, a reduction in outpatient visits was also obtained in adolescents[43,45] and in adults who used home-based tests to measure FC[50]. In a pilot study performed in France with the EasyMICI-MaMICI® platform, a reduction in outpatient visits was also associated with a significant improvement in QoL and satisfaction[51].

Our study group evaluated the impact on health outcomes of the telemonitoring web platform TECCU (Telemonitorización de la Enfermedad de Crohn y Colitis Ulcerosa or Telemonitoring of Crohn's Disease and Ulcerative Colitis), as compared to standard care and telephone care. In a 3-arm randomized clinical trial, 63 patients (21 per arm) with complex IBD were managed with each follow-up method over 24 wk. At the end of the study, the percentage of patients in remission was higher in the TECCU group (17/21, 81%) compared to nurse assisted telephone care (14/21, 66.7%) and standard care (15/21, 71.4%). The telemonitoring group had more improvement in disease activity, and this was associated with a larger reduction in FC values. All completers adhered to treatment in the TECCU group, while QoL, social activities, and satisfaction improved in all 3 groups[52].

Telephone and e-mail support in web-systems

Telephone and e-mail are resources attended by both medical doctors and specialized nurses in some IBD units, with high capacity to solve problems at less cost[53-55]. These tools have also been used to coordinate action plans in telemonitoring systems.

In Spain, the Crohn´s and Colitis Care Unit model has been used since 1999 as a multidisciplinary model of continuous care for patients with IBD. This model manages health demands with distance management mainly through telephone or e-mail with the support of a web page, which includes educational elements. The number of users has risen over the years, with a reduction of in-person care [56]. In Illinois, the Sonar Project is based on monthly web monitoring of symptoms in patients with IBD. Nurses exert a central role and use telephone contact for those patients who send results out of normal ranges, and together with medical health providers management adjustments are performed. This system also reduced hospitalizations, emergency visits and costs[57].

Therefore, beyond the use of telephone and e-mail in units which work as centres for resource coordination, telemedicine in IBD is expanding through the use of web and mHealth systems. These include telemonitoring, tele-education and videocalls in some cases. Its application allows the development of projects to provide health resources in remote areas[58,59], mainly with the use of mobile apps and the integration of some of these platforms into the electronic medical records, as is the case of the app HealthPROMISE and mynexuzhealth[60,61]. These models promote collaboration and mentoring between specialists, which could reduce variability in medical practice and modify the structure of future health systems if they demonstrate to be cost-effective.

Cost-effectiveness of telemonitoring IBD

Although many data about cost-savings have been published, they refer almost exclusively to direct costs[22,27], without considering costs of installation and maintenance of platforms or indirect costs.

In the IBD setting, our research group published the first cost-effectiveness and cost-utility analysis of a telemonitoring program compared to telephone and standard care[62,63]. The differences between groups and statistical uncertainty in disease activity, quality-adjusted life-years, and costs were calculated using nonparametric bootstrap estimations. Even though our trial only included 63 patients, we imputed the original dataset 5 times, and the bootstrapping estimations allowed us to extract 1000 random samples (of 21 patients per arm) from each of the 5 imputations, thus generating 5000 bootstrap replications.

We concluded that there is a high probability (79.96%) that the use of the TECCU web platform for telemonitoring complex IBD patients produces a greater improvement in disease activity at a lower societal cost, compared with standard care. Telemonitoring through the TECCU platform saved €2250 per additional patient in remission (95%CI: €–15363 to 11086) *vs* telephone care, and telephone care saved €538 compared with standard care (95%CI: €–6475 to 5303). Moreover, the use of the TECCU platform and telephone care showed an 84% and 67% probability, respectively, of producing a cost saving per additional quality-adjusted life-year (QALY) compared with usual care, even considering the simulations that involved negative incremental QALYs.

With a similar methodology, our results were reproduced by de Jong *et al*[64] who concluded that telemedicine with myIBDcoach is cost-saving and has a high probability of being cost-effective, without a decline in QoL. In this study, telemedicine resulted in lower mean annual costs of €547/patient (95%CI: €1029-2143) without changing quality adjusted life years. At the Dutch threshold of €80000 per quality adjusted life year, the intervention had an increased incremental cost-effectiveness over standard care in 83% of replications.

The authors included all subtypes of IBD, whereas our study with the TECCU platform recruited complex patients with IBD who needed to start immunosuppressants and/or biologic agents. According to our conclusions, the big sample size recruited in this article is useful to confirm our prior results, and

the reproducibility of the favourable cost-effectiveness profile of telemedicine applied in IBD across countries and patients' characteristics. In fact, during the COVID-19 pandemic, similar cost-savings with a higher gain of QALYs have been observed with the use of telemonitoring for IBD in Hong Kong[65].

ENABLERS AND BARRIERS FOR THE IMPLEMENTATION OF TELEMONITORING IN IBD

Unlike the use of ICTs in other fields (streaming entertainment services, grocery delivery, e-banking, *etc.*), telemonitoring interventions deal with a series of barriers which hinder their definitive implementation to reorganize health systems, despite other associated advantages (Table 2)[66-71]. The factors which favoured and limited these changes can be classified in 5 groups: technological, organizational, legal, acceptability and costs[72].

MODELS OF TELEMONITORING IN IBD

Telemonitoring theoretically include three different diagnostic models: patient self-diagnosis, remote providers´ diagnosis and computer-assisted diagnosis. They usually work as triage systems but, beyond diagnostic capabilities, telemonitoring platforms allow for remote management of other aspects such as disease treatment or education. In the IBD setting, they usually combine self-management, remote providers´ management and computer-assisted telemanagement[10,11,21,22,24,25,42,43,45,47,48,50-52].

Patient self-management

Self-management refers to a dynamic, interactive, and daily process in which individuals engage to manage a chronic illness[73]. This process includes the ability to deal with their own symptoms, treatment, physical and social consequences, and lifestyle changes to maintain a satisfactory QoL[74]. In this sense, telemonitoring platforms for IBD have incorporated PROMS and home-based tests that allowed patients to self-report their health status. This information has been even used to guide treatment adjustments by themselves[22].

Resources for patients´ self-management: PROMs, home-based tests and wearable devices

Considering the "treat-to-target" strategy, the Selecting Therapeutic Targets in Inflammatory Bowel Disease (STRIDE) project recognized different evidence and consensus-based recommendations to optimize outcomes in patients with IBD. Among the different targets proposed, clinical remission and endoscopic healing were confirmed in the STRIDE-II actualization, while normalization of serum and fecal markers of inflammation have been determined as short-term targets[4]. There was agreement to evaluate disease remission with clinical indexes, PROMs and endoscopic criteria [or also radiologic criteria in Crohn's disease (CD)].

Usually, endoscopic disease activity has been considered the gold standard to measure inflammation and to consider mucosal healing, but endoscopy is invasive and expensive.

In this sense, with the aim of measuring inflammation non-invasively, different PROMs and PoC tests have been developed over last years. Moreover, some of these tools have been specifically validated for their use in telemedicine programs.

PROMs: A PROM is a measurement of any aspect of a patient's health status that comes directly from the patient, without the interpretation of the patient's responses by healthcare providers and without the need of laboratory tests[75]. PROMs are designed for screening of disease activity, and then they need to be sensitive enough, especially if it implies more false positive results.

The Simple Clinical Colitis Activity Index (SCCAI) has shown a high correlation and good agreement between patient and clinician reported versions[76]. Compared to UC, PROMs used in the context of CD have shown worse correlation with other markers of clinical or endoscopic activity. The Harvey-Bradshaw index (HBI) had high correlation but only moderate agreement between versions registered by the patient or the clinician[77], although a recent version of the HBI self-administered by the patient through a mobile app showed a high percentage of agreement with in-clinic physician assessment, with a remarkably high PPV for remission[78]. Both SCCAI and HBI show good agreement between paper and online versions[78-80], and represent attractive tools for telemonitoring IBD.

Few PROMs have had their correlation with endoscopic activity evaluated. The global assessment of the patient, based on an analogic visual scale about how they felt regarding their UC during the previous 2 d, only showed moderate correlation with endoscopic activity[81]. The subscore of the global medical assessment and the 6-point Mayo index (which includes stool frequency and rectal bleeding) have a high correlation with the whole Mayo index[82]. Moreover, the 6-point Mayo index has an AUC of 0.80 compared to the endoscopic subscore[83].

Recently, the mobile Health Index was validated to monitor IBD activity through mHealth systems. In patients with CD, it showed high correlation and agreement with the Crohn´s Disease Activity Index and the HBI, as well as in patients with UC when it was compared to the partial Mayo index[84]. The

Table 2 Enablers and barriers for the implementation of telemonitoring in inflammatory bowel disease

	Enablers	Barriers
Technological	Adequate support	Lack of EMR integration
	Sufficient training	System maintenance required to avoid malfunction
	Fast internet connections	
	5G network	
Organizational	Continuous monitoring	Multidimensional nature: complex comparability between programmes
	Overcome geographic barriers	
	Safe assistance during COVID-19 pandemic	Lack of robust data: small studies, short-term follow up periods
	Structured data collection	
	Favours experimental studies and epidemiological surveillance	Lack of standardized remote medical practice (Interstate Mecial Licensure compact in the United States)[66]
	Multicentric access to data	Reimbursement limitations
	Telementoring: professional support and education	
Legal		Lack of legal framework[67,68]
		Data security
Acceptability/accessibility	Patient empowerment	Technological knowledge[69-71]; Some demographic factors increase the likelihood of a telematic encounter failure
	Wide use of smartphones	High drop-out rate in some clinical trials
	Wide use of wearable devices	
	Cheap internet plans	
Costs	Potential decrease of direct and indirect costs	High initial investment
		Limited cost-effectiveness data

intraclass correlation coefficient for test-retest reliability was high for CD and for UC. Nevertheless, its agreement with endoscopic scores was poor in CD and moderate in UC.

QoL and absence of disability are other targets of the STRIDE-II initiative. The Inflammatory Bowel Disease Questionnaire (IBDQ) was specifically validated in patients with IBD and has a moderate to high correlation with treatment response and the endoscopic Mayo index. However, their 32 and 36 items versions require a lot of time for their interpretation, so the reduced versions of 9 and 10 questions were subsequently validated[85,86]. On the other hand, the IBD disability index predicts active disease, nonadherence, and treatment with corticosteroids when high disability values are obtained[87]. Finally, health-related fatigue was incorporated in the Monitor IBD At Home index, but it is still not considered a specific target.

Probably the accuracy of PROMs increases when used in combination with FC. Thus, the Monitor IBD At Home index was developed to predict the endoscopic activity in patients with IBD. The association of FC to both the CD and UC versions showed high sensibility and NPV to rule out endoscopic activity[88]. The development of home-based FC tests that can be measured by the patient represent a potential option to measure disease activity in telemonitoring programs.

PoC tests and home-based tests: The use of PoC tests refers to patient specimens assayed at or near the patient with the assumption that test results will be available instantly or in a very short timeframe to assist caregivers with immediate diagnosis and/or clinical intervention[89]. In the IBD setting, the interest has centered on FC, and even though lactoferrin tests have been developed with adequate accuracy[90], FC offers better sensibility at certain cutoffs[90,91].

FC has good correlation with endoscopic activity in both UC[92,93] and CD[94-96]. FC helps to differentiate between functional and inflammatory diseases in patients with digestive symptoms. Moreover, in patients already diagnosed of IBD it allows the evaluation of disease activity, response to treatment, post-surgical recurrence and it predicts relapses after the withdrawal of anti-TNF agents[97,98]. These features, its non-invasiveness and a relative low cost makes FC tests in a useful tool in the diagnosis, monitoring and treatment adjustment in IBD.

Furthermore, the diagnostic accuracy of FC in different clinical scenarios has increased the interest in its use in telemonitoring IBD programs. In line with a patient-centered care and to favour empowerment, during the last years and the COVID-19 pandemic, different home-based FC tests have

been developed as an additional tool for a home-based follow-up[99]. These tests are based on kits that analysed faecal samples through immunochromatography. Then, the results are read with a smartphone camera, and they are sent through a specific app to a server accessible by providers (Figure 2).

Comparison between different home-based FC tests: Three main FC tests have been developed for its use by patients at home: CalproSmart, IBDoc and QuantonCal.

In a recent study, these 3 tests were compared with the ELISA method of the same manufacturer [100]. Considering the importance of obtaining good agreement in the low range of FC values (ruling-out disease activity), IBDoc, QuantonCal and CalproSmart have an 87%, 82% and 76% agreement, respectively, compared with their corresponding ELISA readings.

However, and similarly to its validation study[101], CalproSmart showed a trend to overestimate FC values with a mean bias of +141 μg/g (95%CI: -316 to 598 μg/g), while IBDoc tended to underestimate FC values with a mean bias of -105 μg/g (95%CI: -576 to 366 μg/g). This could generate more false positive results when using CalproSmart, but misclassification in the low (< 250 μg/g), medium (250-500 μg/g) or high (> 500 μg/g) range of FC values with this test showed large differences (*i.e.*, to classify as > 500 with CalproSmart and < 250 with ELISA, and viceversa) in only 2% of measures, compared to 5% in IBDoc and 8% in QuantonCal.

In any case, the error range between FC measured with home-based tests and their corresponding ELISA method was high. This happens especially when FC values are > 500 μg/g. With values ≤ 500 μg/g, differences were also over the acceptable range of 200 μg/g (+/-100 μg/g), but they were not wide enough to induce errors in the interpretation of inflammatory activity. Therefore, home-based tests are considered useful to rule-out inflammation at a distance when FC values are < 500 μg/g, but when values are > 500 μg/g disease activity should be evaluated with other methods.

Home-drug monitoring: Home therapeutic drug monitoring of monoclonal antibodies appears to be an innovative possibility to improve and simplify IBD management. To date, these tests are performed only in some hospital laboratories and results are not immediate. This delay, of months in some cases, impairs drug monitoring and dose adjustment, compromising its utility.

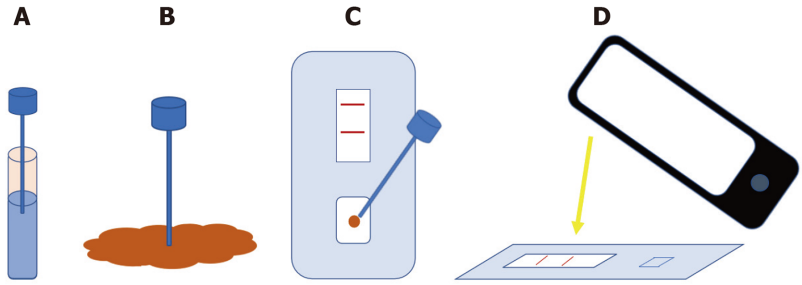
To solve this issue, home drug monitoring using dried blood samples is being evaluated in different inflammatory diseases. The first data were published by Kneepkens *et al*[102] in patients with rheumatic inflammatory diseases. Adalimumab and anti-adalimumab antibodies concentration measurements in finger prick dried blood spots were compared with simultaneous serum measurements. They found that both drug levels and antibody concentrations from the finger prick method correlated well with serum measurements (correlation coefficient > 0.87). However, some disadvantages should be considered, such as loss in precision, workload and elevated costs.

Berends and colleagues did a similar study with 40 IBD patients, comparing infliximab concentrations in dried blood samples and serum. Home-based test infliximab concentrations showed a good correlation (correlation coefficient: 0.671) with serum measurements[103]. This author also published data with adalimumab treatment one year later. A high correlation was found (Pearson's correlation: ≥ 0.96) between dried blood test and venipuncture results when performed at the same time during the outpatient clinic. Moderate correlation (Pearson coefficient = 0.51) was reported between home self-performed finger test and estimated adalimumab concentrations[104]. Larger studies are needed to confirm the reliability, accuracy, and cost effectiveness of home-based testing as a new telemonitoring tool in IBD.

Wearable devices: Wearable devices are electronic gadgets that consumers wear to track health-relevant physiological data to monitor and improve health[105]. IBD patient preferences and interest in wearable technology were evaluated by Hirten and colleagues using a 28-question survey. Four hundred patients completed the survey. Of these, 42.7% reported prior or current use of wearable devices, mainly smart watches (34.5%) and wrist band devices (29.1%). Almost 90% of subjects believed these gadgets could provide important information about their health and 93.8% reported that they would use them if it could help doctors manage their disease[106].

In the last few years, several studies have tried to demonstrate the utility of wearable devices in IBD telemonitoring[107]. The first available data was published by Yvellez *et al*[108] in 2018. They prospectively assessed daily health-related QoL, pain and sleep data using validated indexes through a mobile application and a Fitbit® device. Fitbit® compliance was almost 80%, suggesting this technology is feasible[108]. The Fitbit® device has also been used to predict disease activity. In one study a significant reduction in daily steps has been shown over the week before CRP or FC elevation, but without differences in daily resting heart rate[109]. Two years later, however, Hirten *et al*[110] demonstrated that significant changes in heart rate variability measured using VitalPatch® were observed before the development of symptomatic or inflammatory flare in ulcerative colitis patients.

Step count and sleep monitoring have also been used, not to predict flares but the intent to determine post-operative length of stay. Overall, step count and sleep duration/efficiency did not predict length of stay. However, in a multivariable linear regression model, significant interaction was found between postoperative complications and step count, suggesting that increased physical activity was associated with a reduction in duration of hospital stay[111].



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Figure 2 Procedure to measure fecal calprotectin at home. A: Extraction buffer; B: Collection of faecal sample; C: Control (C) and test (T) lines appear after application of extracted calprotectin to the sample window; D: A smartphone camera is used to read the result obtained.

Remote providers´ management

All the telemonitoring interventions in IBD reported in this review comprise healthcare providers´ advice. Most systems employ store and forward programs, where nurses acquire a central role in tracking the information received and making contact between patients and specialists to set up healthcare plans. Communication is established through websites, usually with the support of telephone and e-mail, as reported above.

Computer-assisted telemanagement

Computerized systems have been used for telemonitoring IBD and they usually work as a triage system to identify which patients might require further evaluation. Many of them can generate automatic action plans through the integration of different monitorable indicators in decision-making algorithms. In most telemonitoring programs tested thus far, these tools are combined with self-management and the remote providers´ management models seen above.

Telemonitoring systems in IBD are integrated by personal computers or mobile devices used by patients, a decision support server and a website for staff and providers. The website provides an interface to collect data from testing sessions, and these platforms usually generate automated reminders to favour adherence to follow-up. The structure of most eHealth tools in IBD are based on a traffic light system[10,11,22,25,27,43,45,52,112]. Patients usually enter their symptoms in scheduled online controls, mainly in a structured manner through PROMS, but many apps also include a comment box to freely express anything outside the questionnaires. Then, the patient´s status appears as red, yellow or green when disease is highly, moderately active, or quiescent, respectively.

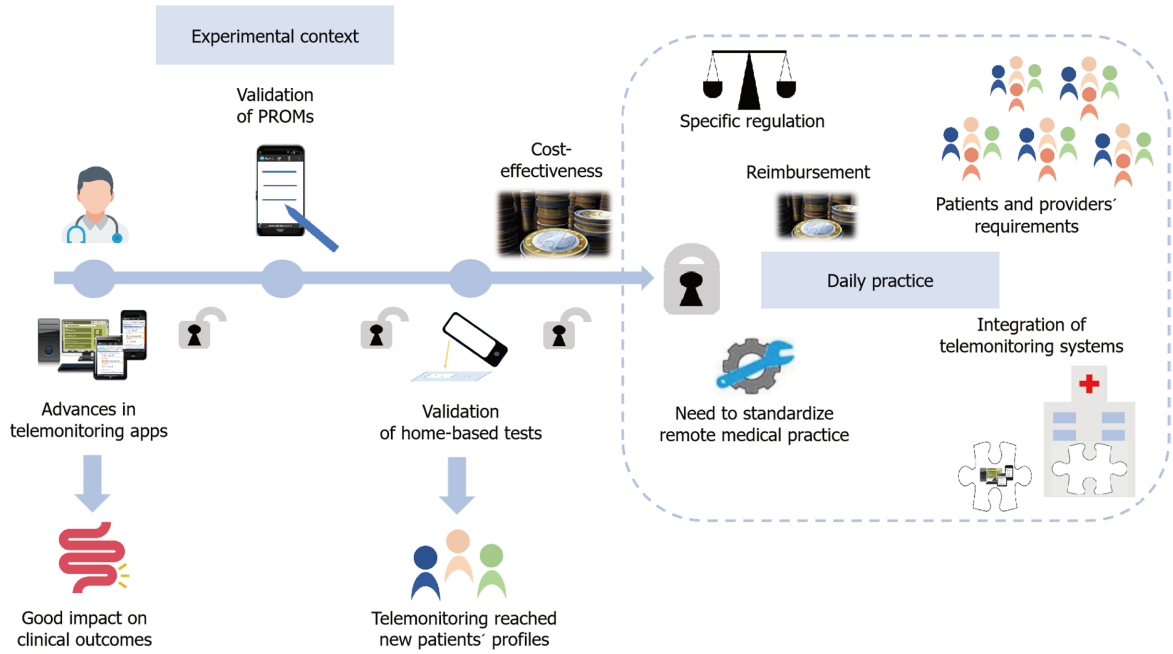
Some systems combine self-reported symptoms with the level of FC in a total inflammation burden score. In fact, recent clinical trials have incorporated FC tests performed by patients at home[45,50,112]. This status is sometimes supplemented with disease activity and QoL graphs[10,11,22]. Depending on the level of alert, simultaneous action plans and email alerts are sent to the participant and providers, who review the information to decide if further management changes are necessary.

Altogether, these new models and resources for patients´ self-management represent advances to reach the implementation of telemonitoring in daily practice, but still some legal, ethical, economical and logistic barriers need to be solved (Figure 3).

DISCUSSION

Telemonitoring is about communication, and the development of faster and wireless systems at a lower cost has supported the use of proactive remote monitoring. As well, the increase in data storage also favoured the incorporation of tele-education in most telemonitoring programs in the IBD setting. However, during the pandemic, e-mail and telephone still represented the main resources used[29,30], while the application of mature telemonitoring programs was the exception. As different enablers encouraged advances of telemonitoring in IBD, many other barriers emerged and hindered its full implementation in daily practice.

Pioneering studies evaluated telemonitoring programs previously used in other chronic diseases[21,42,113,114]. Feasibility and patients´ acceptance of these applications was excellent. Yet, they were not able to clearly demonstrate an improvement in QoL, disease activity, and treatment adherence. These systems were based on remote monitoring through computers, they needed to be adapted to the IBD context and required eventual repairs at home, which were time expensive. Telemonitoring subsequently evolved towards the use of web-based systems, which were cheaper and easy to use. Remote monitoring through the web demonstrated its feasibility and excellent patients' acceptance, with an improvement in QoL, disease activity, and disease knowledge[22,115].



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Figure 3 Advances in telemonitoring of inflammatory bowel disease related to the enablers and barriers for its implementation in daily practice. PROMs: Patient reported outcome measures.

In the last few years, telemonitoring prioritized the use of mHealth resources[24,25,116]. Beyond the improvement in clinical outcomes, mHealth telemonitoring associated cost-savings in outpatient visits and hospitalizations[23-26]. Almost parallel to the mHealth evolution, many PROMs have been validated to self-report disease activity. In line with patient-centered care, empowered patients can use new home-based calprotectin tests, which are accurate enough and useful to rule-out disease activity at low FC values[100,101]. In fact, new home drug monitoring is being developed to measure levels of monoclonal antibodies near the patient[103,104]. Furthermore, the development of mobile devices even enabled the increasing use of wearables to monitor physiological predictors of disease activity and postoperative length of stay[110,111]. These new tools could represent one of the first steps towards ubiquitous Health in IBD, and in a near future machine learning may allow the integration of large data sets in personalized algorithms.

Despite the technological and knowledge advances reached, the effect of telemonitoring on health outcomes is not consistent in different populations and health systems[22,24,25,42,44,45,52]. Initially, remote monitoring was mostly restricted to UC patients, while the design of new apps, PROMs and home-based tests allowed to progressively expand its use to a broad range of patients´ profiles. However, telemonitoring has not been demonstrated to improve QoL or clinical/endoscopic remission in the long-term. In addition, biomarkers in IBD are less accurate compared to other chronic diseases such as diabetes mellitus, and the early recognition of complications in IBD still require invasive tests in many cases.

On the other hand, although telemedicine has been traditionally considered cost-effective, cost-saving data previously published referred almost exclusively to direct costs[22,27]. The implementation of telemonitoring services represents a short-term high initial cost, not only from a technological point of view, but also by changes in the organization of the IBD units. Thus, decision-makers have had difficulties to support the implementation and investment in telemedicine due to a lack of solid evidence so far. In addition, these decisions become even more complicated in areas where reimbursement is an important factor in the setup of clinical activity. In this regard, recent studies suggested a good cost-effectiveness profile of telemonitoring[62-65], even after considering the costs of installation and maintenance of platforms, as well as indirect costs.

The availability of more powerful and cheaper communication tools turned technical challenges into legal, ethical, economical, and logistic issues[28]. To standardize remote medical practice, in the US the Interstate Medical Licensure Compact was created to increase efficiency in multistate licensing of physicians[66] but such a proposal is lacking in Europe. Besides, only a few examples of full integration of telemonitoring programs into electronic medical records are available to date[60,61]. In this sense, interoperability of systems while maintaining the confidentiality of data cannot be guaranteed in many centres. Moreover, the provision of remote health safely also requires a specific European regulation to protect remote medical practice and to lift some existing legal barriers. Finally, to keep adherence to follow-up, it is essential to adapt telemedicine programs according to patients' and providers´ character-

istics, because some demographic factors such as increasing age, commercial insurance status and racial differences increase the likelihood of a telematic encounter failure in some contexts[117].

CONCLUSION

Therefore, telemonitoring IBD is well accepted and improves clinical outcomes at a lower cost in the short-term. The advances performed on new PROMS, home-based tests and wearables improved the ability to manage new patients´ profiles remotely. However, it is still necessary to overcome many legal, ethical, economical and logistic barriers. Funders, policymakers, providers and patients need to align their interests to successfully implement telemonitoring, and further collaborative efforts based on teamwork between centres are essential to help reorganize health systems.

FOOTNOTES

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Country/Territory of origin: Spain

ORCID number: Javier Del Hoyo 0000-0003-3603-5793; Mónica Millán 0000-0001-5321-1432; Alejandro Garrido-Marín 0000-0003-1299-9010; Mariam Aguas 0000-0003-4353-569X.

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REVIEW

Mucosal healing and inflammatory bowel disease: Therapeutic implications and new targets

Megan Lynn Otte, Raju Lama Tamang, Julia Papapanagiotou, Rizwan Ahmad, Punita Dhawan, Amar B Singh

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Megan Lynn Otte, Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE 68198, United States

Raju Lama Tamang, Julia Papapanagiotou, Rizwan Ahmad, Punita Dhawan, Amar B Singh, Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE 68198, United States

Corresponding author: Amar B Singh, PhD, Professor, Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, 600 42nd Dewey, Omaha, NE 68118, United States. amar.singh@unmc.edu

Abstract

Mucosal healing (MH) is vital in maintaining homeostasis within the gut and protecting against injury and infections. Multiple factors and signaling pathways contribute in a dynamic and coordinated manner to maintain intestinal homeostasis and mucosal regeneration/repair. However, when intestinal homeostasis becomes chronically disturbed and an inflammatory immune response is constitutively active due to impairment of the intestinal epithelial barrier autoimmune disease results, particularly inflammatory bowel disease (IBD). Many proteins and signaling pathways become dysregulated or impaired during these pathological conditions, with the mechanisms of regulation just beginning to be understood. Consequently, there remains a relative lack of broadly effective therapeutics that can restore MH due to the complexity of both the disease and healing processes, so tissue damage in the gastrointestinal tract of patients, even those in clinical remission, persists. With increased understanding of the molecular mechanisms of IBD and MH, tissue damage from autoimmune disease may in the future be ameliorated by developing therapeutics that enhance the body's own healing response. In this review, we introduce the concept of mucosal healing and its relevance in IBD as well as discuss the mechanisms of IBD and potential strategies for altering these processes and inducing MH.

Key Words: Inflammation; Injury/repair; Mucosal healing; Mucosal barrier; Therapeutics; Colitis

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Core Tip: Mucosal healing (MH) is vital in maintaining intestinal homeostasis and protecting against infection and injury. MH has emerged as an important clinical criterion in effective treatment of inflammatory bowel disease (IBD). However, there remains a relative lack of therapeutics that can restore MH due to the complexity of the disease and healing processes. Through increased understanding of the molecular mechanisms of MH, tissue damage from IBD may be ameliorated by developing novel therapeutics. Here, we introduce the concept of MH and its relevance in IBD, and discuss the mechanisms of IBD and potential strategies for altering these processes for inducing MH.

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INTRODUCTION

Mucosal healing (MH) is a process of wound repair that restores the integrity of damaged epithelial barrier and homeostatic function after an injury compromises barrier integrity[1]. It is a complex process regulated by multiple cell types, through distinct mechanisms in response to highly specific stimuli within multiple signaling and cytokine pathways[1]. For simplification, the MH process is considered to have three phases: Epithelial restitution, proliferation, and differentiation and maturation[1]. Restitution consists of epithelial cells migrating into a wound within hours, followed by proliferation of epithelial cells in hours to days, and finally differentiation of intestinal stem cells into all mature intestinal cell types[1-3]. Each phase is induced and regulated by multiple cytokines, growth factors, and cell types reviewed in[1] and can be influenced by many factors that enhance or prevent wound healing as well as by the source of barrier injury. When the homeostatic process of wound healing is slowed or delayed by external or genetic factors, chronic inflammation may develop because repair of the intestinal epithelial barrier (IEB) and subsequent reduction of inflammation will not occur unless wound healing mechanisms are present[3]. Due to inflammation and chronic immune response, the consequences of impaired MH are chronicity of autoimmune diseases, including Inflammatory Bowel Disease, (IBD) and its progression to colorectal cancer.

INFLAMMATORY BOWEL DISEASE OVERVIEW AND PATHOGENESIS

Inflammatory Bowel Disease (IBD) is a term that represents autoimmune inflammatory diseases of the gut, with ulcerative colitis (UC) and Crohn's Disease (CD) being the major disease types; however, the etiology of IBD remains unclear. IBD is known to have genetic and environmental risk factors, but the mechanisms by which these factors induce IBD are not well-understood[4]. Common symptoms include abdominal pain, diarrhea, weight loss, malnutrition, and particularly in CD, nausea, vomiting, intestinal blockages, fistulae, and abscesses[4,5]. IBD is an increasingly common and often debilitating disease, affecting up to 200 individuals *per* 100000 people in the United States[4]. Onset of IBD often occurs before the age of 30, and patients experience poor quality of life along with high risk of developing colorectal cancer due to the chronic and progressive symptoms and persistent inflammatory state[1,4]. Though multiple treatment mechanisms exist, including corticosteroids, anti-inflammatory medications, monoclonal antibodies, stem cell treatments, and surgery, IBD cannot currently be cured[4]. Therefore, continued research into the mechanisms of pathogenesis and development of new pharmaceuticals and treatment methods is vital to decrease mortality and improve quality of life for IBD patients.

Autoimmune disease pathogenesis is difficult to study due to the multifactorial causes of disease and complex molecular mechanisms, as well as the predicament of patients not presenting to the clinic until late in the disease development process. Although the disease complexity and difficulty in identifying the initial molecular instigators has thus far precluded full understanding of the specific molecular mechanisms of IBD pathogenesis, the general overarching processes have been elucidated. During IBD pathogenesis, the IEB and mucosal layers become damaged and inflamed due to injury and/or infection, which can develop into a state of chronic inflammation and reduced IEB integrity. Genetic factors can also play a role in pathogenesis, particularly in CD through genes such as a variant that impairs autophagy and dysregulates the IEB and gut microbiota[6,7]. The damage to the IEB results in microbial and antigen exposure in the intestinal lumen, leading to an inflammatory cascade and disturbed homeostasis[8,9]. Multiple cytokines and immune cell types are also thought to contribute to the pathogenesis of or protection against IBD; therefore, further study of the complex interactions governing the maintenance or breakdown of gut barrier homeostasis is imperative to deeper

understanding of IBD pathogenesis and the development of effective treatments.

MUCOSAL HEALING AND PATHOLOGICAL RELEVANCE IN IBD

The main goals of IBD treatment are two-pronged: Reducing symptoms and preventing new inflammation and intestinal injury through traditional treatment methods, and recently a new, ambitious goal of inducing wound healing of existing inflammation and damage[8]. Some current treatments may contribute to both treatment goals; however, many existing clinical treatments are more targeted toward the traditional goal of preventing inflammation and damage. Even with the advent of some newer MH-focused treatments, additional avenues of further enhancing MH should be explored. Although clinical remission or preventing new inflammation is possible, patients may still have residual disease symptoms during remission due to the defective wound healing process leaving previous intestinal damage unrepaired. Additionally, up to half of IBD patients experience non-response or loss of response to standard therapeutics, leading to relapse[10]. Hence, MH induction presents an attractive goal in effective long-term treatment of IBD and prevention of relapse, and thus also prevention of progression to colitis-associated colon cancer. In this review, we will summarize the main current and prospective treatments of IBD, as described in Table 1, and their benefits and limitations towards the goal of reducing inflammation and achieving MH. More detailed analysis of the mechanisms of action, safety, and efficacy profiles of current IBD therapies and clinical trials can be found in Neurath *et al*[11]. However, in this review, we will emphasize areas that have not yet been as extensively clinically explored, including the temporal control of gut immune function, which presents novel potential for fine-tuning of the immune system in MH and restoration of gut homeostasis. We will examine three major factors contributing to the pathogenesis and tissue damage of IBD, as shown in Figure 1: Gut barrier dysfunction, gut dysbiosis, and inflammatory cytokine responses. Specifically, we will focus on the prospect of altering these factors and associated pathways summarized in Table 2 for both the reduction of inflammation and induction of MH.

CURRENT STATUS OF IBD THERAPIES AND FOCUS ON ENHANCED MUCOSAL HEALING

Traditional Therapies

Historically, there has been a disconnect between the expectation of IBD treatment promoting MH and real treatment outcomes, as many therapies for UC and CD primarily target symptom relief and reduction of chronic inflammation[12]. Corticosteroids, Methotrexate, and surgery are typically utilized to achieve these goals, but they do not promote MH as the primary therapeutic endpoint[13,14]. Because UC and CD are progressive diseases, patients may still experience intestinal damage even during periods without physical symptoms, and disease progression is typically only slowed by these treatments, not stopped[12,15]. However, achieving MH may help stop disease progression as well as decrease symptom severity. New treatment plans broaden the therapeutic focus to include inducing MH through a variety of mechanisms, such as by altering the gut microbiome and altering inflammatory and anti-inflammatory cytokines with antibodies or exogenous cytokine therapies.

Inflammation Reduction and Immune Modulation Therapies

One such mechanism for emphasizing MH includes suppressing specific parts of the patient's immune system to decrease the main contributors of chronic inflammation. When new inflammation is reduced, it may then be possible for the wound healing process to begin to keep up with the rate of tissue damage. Methods of achieving this goal include enteral nutrition (EN), partial EN (PEN), 5-aminosalicylates (5-ASA), and Azathioprine treatments, which focus on decreasing IBD-associated inflammation by suppressing the host immune system in a less global manner than corticosteroids or preventing the production of inflammatory molecules. EN is a dietary treatment for IBD patients that can either totally (with EN) or partially (with PEN) replace solid food intake with specialized formula[16]. Although the mechanism by which EN induces MH is currently unknown, microbiome changes are implicated and are hypothesized to aid in decreasing chronic inflammation[17]. While typically a pediatric treatment, a pilot study shows MH in adults following EN and PEN treatment as well[16]. Currently, the largest drawback of EN treatment is low patient compliance, especially in adults[16]. 5-ASA drugs, which are used to induce remission in early IBD, are shown to induce MH in 43.7% of patients[18]. Azathioprine is a purine analog inhibiting purine metabolism and blocking T cell activation and co-stimulation, therefore functioning by suppressing the immune system and decreasing inflammation in IBD[11,19-21]. It is shown to achieve MH independently in some cases (30.1%) alone but is more successful when used in conjunction with anti-TNF-α antibodies such as Infliximab (44%)[22]. Other immunomodulating drugs shown to induce MH when given with monoclonal antibodies include Cylosporine and Tacrolimus[23].

Table 1 Summary of inflammatory bowel disease therapeutics

Treatment type	Available therapeutics	Mucosal healing relevance/ Success
Corticosteroids	Prednisone/ Prednisolone/ Methylprednisone	Prednisone treatment for 14 d (20 mg/day) decreased mucosal inflammation indicating a possible role in developing short-term MH[139]. 29% of patients in one study displayed endoscopic remission after steroid treatment[140].
Nutritional therapy	Enteral nutrition (EN)/ Partial enternal nutrition (PEN)	EN/PEN induce MH in both adults and children[14].
Aminosalicylates (5-ASA)	Sulfasalazine/ Mesalamine/ Olsalazine/ Balsalazide	On average induce MH in 43.7% of patients[141].
Immunomodulators	Azathioprine/ 6-mercaptopurine	Azathioprine alone has achieved MH in 16.5% of cases and in 43.9% when used in combination with antibody therapies[18]. After 16 wk of mercaptopurine treatment, patients in remission showed a 47.1% rate of MH[142].
	Cyclosporine	Shown to induce MH when used in conjunction with Vendolizumab[143].
	Tacrolimus	Shown to induce MH when used in conjunction with Vendolizumab[143].
	Methotrexate	After 36 wk, methotrexate treatment had a MH rate of 47.4%[142].
Monoclonal antibody/ Biologic therapies	Adalimumab	Induced MH in 24% of patients treated[24].
	Certolizumab	Clinical response rate at weeks 2 and 12 was 29.7% and 52.8% (respectively) in CD[25].
	Infliximab	Treatment induced MH in up to 60.3% of patients in phase 2 clinical trials[23].
	Natalizumab	MH achieved by 42.3% of patients after 14.1 mo of treatment[144].
	Risankizumab-rzaa	Endoscopic response and deep remission observed in 55% and 29% of patients (respectively), indicating MH[27].
	Ustekinumab	Treatment of individuals with moderate to severe CD showed MH *via* a reduced disease score after 8 wk[19].
	Vedolizumab	Has shown to induce MH in up to 50% of UC patients and 29% of CD patients in clinical trials[26,27].

MH: Mucosal healing; EN: Enteral nutrition; PEN: Partial enteral nutrition; 5-ASA: Aminosalicylates; CD: Chron's Disease; UC: Ulcerative colitis.

Monoclonal Antibody Therapies

Monoclonal antibodies targeting inflammatory cytokines are an emerging class of IBD therapies that more directly focus on permitting or inducing MH and are an attractive method of IBD treatment. Monoclonal antibody drugs targeting multiple cytokines are approved or in trials, and function by removing inflammatory cytokines. Anti-tumor necrosis factor-alpha (TNF-α) antibody-based drugs such as Infliximab, Adalimumab, and Certolizumab are biologic therapies that have achieved significant clinical success and are now widely used as front-line treatments for IBD, with the goal of reducing inflammation and promoting MH. The target of anti-TNF-α therapies is a pro-inflammatory cytokine contributing to the chronic and severe inflammatory response observed in UC and CD, and the monoclonal antibody functions by downregulating pro-inflammatory molecules and restoring IEB integrity, allowing the body to begin to heal[8,24-26]. Infliximab is an Immunoglobulin G1 anti-TNF-α antibody that is shown to induce MH. Active phase 1 and 2 clinical trials of Infliximab in UC patients find that, respectively, 45.5% of patients and 60.3% of patients exhibit MH[27]. Adalimumab is another anti-TNF-α antibody presenting some indication of MH effects. In the double blind, randomized, placebo controlled clinical trial, 24% of patients display MH at week 52 compared to 0% in the placebo group[28]. Certolizumab pegol is an anti-TNF-α antibody fragment targeting soluble and trans-membrane TNF. Its role in promoting MH is not yet well-investigated, but it demonstrates symptom relief and remission at week 8[29]. In some patients, anti-TNF-α treatments are ineffective or lose efficacy over time. In these patients, biologics targeting other inflammatory cytokines are employed as IBD treatment options. For example, Ustekinumab is a monoclonal antibody that targets interleukin (IL)-12 and IL-23 pro-inflammatory cytokines. Ustekinumab significantly reduces SES-CD (Simplified Endoscopic Activity Score for Crohn's Disease) scores in patients over 44 wk, implying its ability to enhance MH[30]. Similarly, Risankizumab, an IL-23 antibody, induced remission in 45% of patients, with an endoscopic response rate of 29%[31]. Additionally, Natalizumab, an anti-α4-integrin antibody, induces MH in 42.3% of patients[32]. Vedolizumab, on the other hand, is an $\alpha_4\beta_7$ integrin receptor antagonist and biologic medication, with potential immunosuppressive effects localized to the colon. In a clinical trial, 50% of UC patients and 29% of CD patients display MH following long-term use[33,34].

Although inflammation reduction and monoclonal antibody treatments have greatly enhanced the quality of IBD treatment in recent years, lack or loss of response occurs with concerning frequency, and even in patients who respond well to treatments, complete resolution of the disease has not been

Table 2 Summary of molecular pathways involved in mucosal healing

Pathways/ Mechanism of action	Associated models studied	Ref.
EGFR signaling	*In vitro*, colorectal cancer mice, EGFR mutant mice	[43,116]
Hippo/YAP signaling	*In vitro*, YAP-1 transgenic mice	[36,59]
Notch signaling	Villin-Claudin-1 transgenic mice	[41,42]
Wnt/β-catenin signaling	*In vitro* and *In vivo models of injury/repair*	[44,60,61]
Vitamin D receptor (VDR) signaling	*In vitro*, VDR knockout mice	[45]
Src/focal adhesion kinase	*In vitro*, Mechanical colonic wound in mice, Nox1 and AnxA1 knockout mice, oral gavage in mice	[76-78]
Autophagy/ATG16L1	Patient biopsies; ATG16L1 T300A knock-in mice; Atg5-manipulated mice	[6,7,104]
SCFA-mediated signaling [acetate, propionate, butyrate, *etc.*]	*In vitro*, Patient biopsies, oral gavage in mice. T-cell induced colitis, trinitrobenzenesulphonic acid (TNBS) colitis	[83-85,91,93,100,101,114]
TLR-mediated signaling	DSS colitis	[109,110,112]
MyD88 mediated bacterial sensing	Mechanical colonic wound, MyD88 knockout mice	[111]
Prostaglandin-endoperoxidase synthase 2 enzyme (PGE2)	*In vitro*, mechanical colonic wound, Ptgs2 knockout mice, Ptger4 knockout mice	[111,112]
Mucin 2 signaling	*In vitro*, DSS colitis, EGFR mutant mice	[80,116]
IL-6/IL-22/IL-23/STAT3 signaling	DSS colitis, Th2-mediated colitis, cytokine deficient mice, bone marrow transplant mice, T-cell induced colitis, human and mouse intestinal organoid culture	[94,97,98,136-138]
TGF-β signaling	*In vitro*, DSS colitis, TGF-β transgenic mice	[50,130,131]
IL-10 signaling	*In vitro*, mechanical colonic wound in mice, IL-10-deficient mice	[132,133]

EGFR: Epidermal growth factor receptor; YAP: Yes-associated protein 1; ATG16L1: Autophagy related 16 like 1 protein; Atg5: Autophagy related 5; SCFA: Short chain fatty acid; TNBS: Trinitrobenzenesulphonic acid; TLR: Toll-like receptor; DSS: Dextran sodium sulfate; PGE2: Prostaglandin-endoperoxidase synthase 2 enzyme; IL: Interleukin; STAT3: Signal transducer and activator of transcription 3; TGF-β: Transforming growth factor-β.

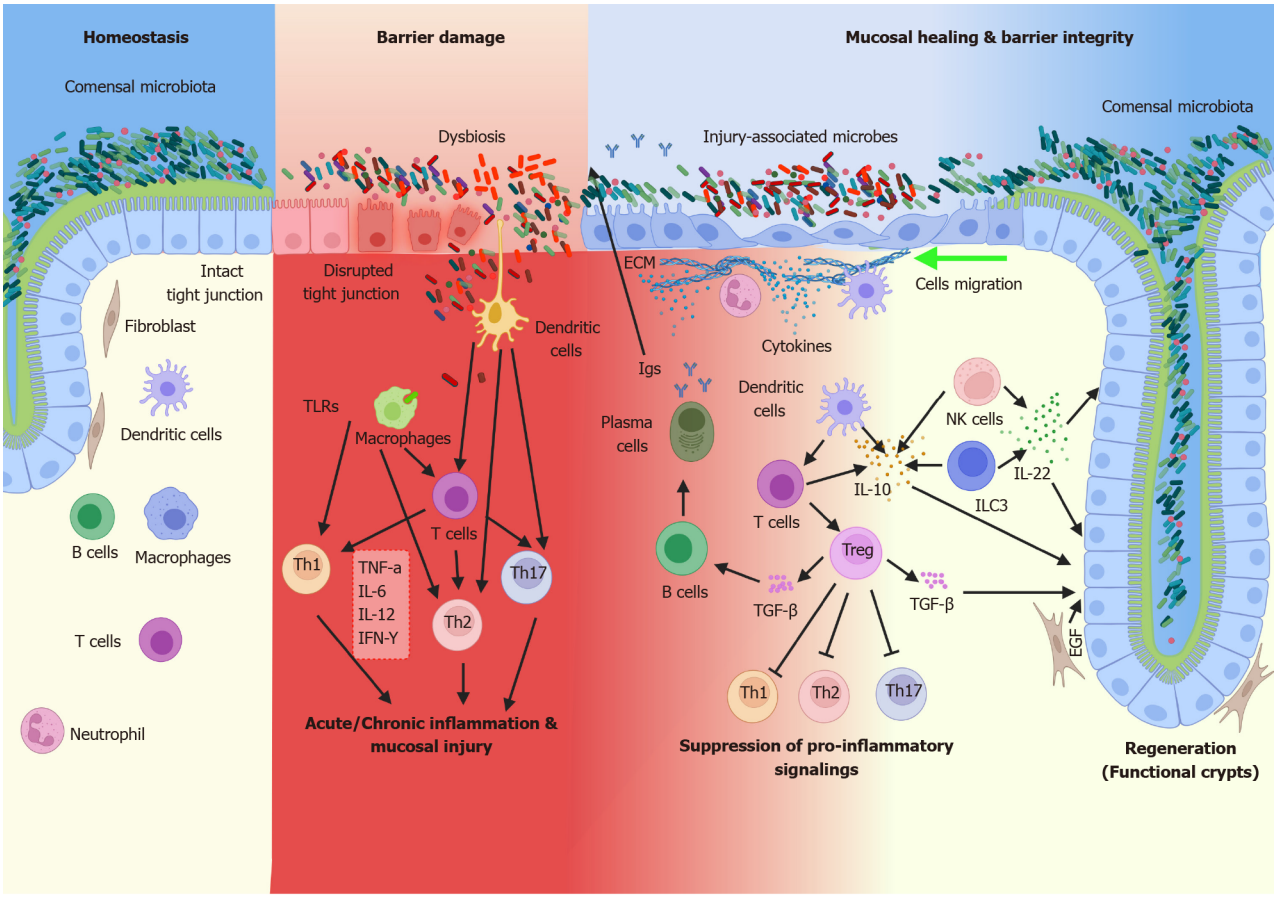
achieved[10]. This is due to the complex nature of disease development through many aberrant proteins and signaling pathways, as well as the multi-faceted process of MH that must be approached from many angles to restore complete IEB homeostasis. Overall, while current therapies offer evidence of permitting MH in IBD, some of these therapies do not directly promote MH through enhancing the processes of restitution, proliferation, or differentiation, but rather by simply inducing immune suppression to decrease inflammation and associated injury. Subsequently, there is a critical need to better understand the processes of inflammation and MH in IBD to aid in the development of actively MH-inducing IBD therapies.

IBD TISSUE DAMAGE MECHANISMS AND IMPLICATIONS FOR MH INDUCTION

Gut Barrier Functions and Mucosal Healing

A major role of the intestinal epithelium is its function as a barrier against the luminal environment and antigens; a role that is critical in maintaining normal mammalian homeostasis[35]. Accordingly, IEB dysregulation is a major factor in gut inflammation; thus, reinforcement of IEB integrity is a key consideration when developing more effective treatments for gut inflammatory diseases, including IBD. The MH process has been shown to help reinforce barrier integrity[36]. A complex and dynamic coordination between epithelial and immune factors facilitates MH, however, the 'paradoxical' role of the barrier-integral proteins in promoting MH remains ill-understood. Here, we summarize the key components of IEB regulation to illustrate the dynamic causal relationship between MH and the proteins comprising the structural and functional units of the gut barrier.

The physical component of the IEB consists of a single layer of epithelium, with cells linked by junctional complexes along the apicobasal axis. Excellent reviews have described the types and roles of junctional complex proteins in barrier integrity, thus here we focus on mechanisms by which regulation of these proteins influences inflammation and MH[35-39]. Tight junctions, the most apical cell-cell adhesions are considered the "gate" of the IEB and consist of multiple proteins, including the Claudin family of proteins, Occludin, junctional adhesion molecule (JAM) and the zonula occludens (ZO)-proteins[40]. Studies in cell and mouse models demonstrate through genetic manipulation that tight



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Figure 1 Pictorial depiction of inter-connection between the immune system, inflammation, and microbiota in mucosal inflammation, associated injury, and healing. Left: normal mucosal homeostasis; Middle: Inflammatory lesions damage the mucosal barrier between the gut lumen and the rest of the body. Barrier damage leads to immune cell activation, cytokine release, and feedback cycles of deteriorating inflammation driven by microbes crossing the damaged barrier; Right: Migration of circulating restitutive immune cells to the wound area, the release of repairing cytokines; crosstalk among extracellular matrix and epithelial cells for proliferation and migration; switching of microbiota and cytokines for mucosal healing and functional crypt regeneration.

junction proteins are not merely static structural entities of the IEB; they perform additional non-canonical roles of regulating epithelial cell proliferation, survival, differentiation, and migration, which are the same processes integral to MH. In this regard, ZO-1 regulates expression and nuclear localization of the transcription factor ZO-1-associated nucleic-acid-binding protein to influence cell proliferation in a density dependent manner[41,42]. Recent studies also show that the ZO-proteins modulate Hippo/Yes-associated protein 1 signaling, a critical regulator of crypt growth and MH[43]. A similar role of JAM in regulating intestinal epithelial cell (IEC) proliferation has been reported[44,45]. Occludin, on the other hand, is shown to regulate IEC apoptosis and survival[46,47]. Recent studies demonstrate that the Claudin family of proteins is integral to tight junction structure and function and plays a complex role in gut inflammation and regenerative processes. For example, Claudin-1 overexpression in the intestinal epithelium of mouse models of IBD induces significant dysregulation of Notch/Wnt signaling and severe colitis, resulting in delayed recovery from colitis-associated injury[48,49]. Claudin-2 is unique among the Claudin proteins expressed in the intestine, as it is primarily expressed at the crypt base among proliferative undifferentiated cells and associates inversely with the differentiation state of IECs[48]. Of note, Claudin-2 is a direct target of Epidermal Growth Factor Receptor (EGFR), Wnt/β-catenin, and Vitamin D receptor signaling, all of which promote intestinal MH [50-52]. Claudin-3 on the other hand, which functions as a receptor for *Clostridium perfringens*, is sharply downregulated in the biopsies of the IBD patients, and loss of its function in the mouse gut promotes colitis-associated cancer[53-55]. We have also previously reported that Claudin-3 Loss enhances gp130/IL-6/STAT3 (signal transducer and activator of transcription 3) signaling, which promotes colitis-associated injury/repair[50]. Interestingly, the loss of intestinal Claudin-7 expression results in spontaneous inflammation due to dysregulation of epithelial-extracellular matrix interactions[56,57]. Claudin-7 also regulates intestinal stem cell function in association with the Epithelial Cellular Adhesion Molecule protein[58,59]. Conversely, Claudin-15 Loss in the IEB results in a mega-intestine[60]. Hence, dysregulation of tight junction protein expression in the IEB results in IBD, indicating that restoration of tight junction homeostasis is a vital component of MH and a promising target for treatment

development.

Like tight junction component proteins, adherens junction proteins contribute significantly to gut inflammation and MH. Here, E-cadherin, a protein whose expression indicates an epithelial phenotype, contributes to inflammation-associated epithelial repair by regulating the epithelial-to-mesenchymal transition, a process associated with cell proliferation and migration[58,61,62]. Specifically, E-cadherin expression inhibits migration of IECs and wound healing[63]. Contrastingly, complete E-cadherin loss causes a severe inflammatory phenotype characterized by villus blunting, which is a marker of premature epithelial death, and incomplete brush border development[64]. The stabilization of E-cadherin expression is facilitated partly by binding with the cytoplasmic domain of p120-cadherin. Accordingly, p120 knockout mice displayed disrupted intestinal integrity and early death from intestinal injury[65]. Other heterodimeric adherens junction proteins, α- and β-catenin, are key players in the regulation of Hippo and Wnt signaling[66,67]. Although α- and β-catenin expression dictates IEC proliferation and differentiation during injury repair, their expression counter-acts each other. Moreover, ubiquitination of β-catenin by α-catenin aids the degradation of β-catenin, balancing the Wnt signaling pathway[68]. Taken together, these findings support a complex and dynamic interdependence between gut barrier regulation and MH, which should be considered for therapeutic potential.

Gut Dysbiosis, IBD, and Mucosal Healing

Aberrant microbiome-immune interactions can lead to improper immune activation and are potentially responsible for the clinical and endoscopic observations in IBD patients. Mechanisms of microbial involvement in IBD include production of short-chain fatty acids (SCFAs), interaction with autophagy pathways, activation of immune cells, TLR signaling, and prostaglandin pathways[69-73]. Excellent reviews have covered the details of such interactions and the dynamic association with gut inflammatory processes[71-75]. Thus, here we focus primarily on how gut microbiota may contribute to MH processes under normal and/or inflammatory conditions.

Gut dysbiosis is mediated by pathogenic microbes harboring genes encoding toxin proteins, which disrupt the IEB *via* disassembly or redistribution of tight junction proteins. For example, human epithelial cells treated with *Escherichia coli* or *Salmonella typhimurium* demonstrate downregulation of ZO-1 and Occludin proteins while by contrast, Claudin-2 is upregulated[76-78]. *Shigella flexneri* and *Campylobacter jejuni* are involved in deregulating E-cadherin, as well as activating IL-8 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), thereby inducing barrier dysfunction and inflammation[79,80]. Overall, studies suggest that pathogenic gut bacteria promote chronic mucosal inflammation by dysregulating IEB integrity.

On the other hand, commensal gut bacteria seem to promote the initial stage of epithelial restitution, as studies in germ-free mice show impaired rates of epithelial cell migration, which is dependent on the formation of focal adhesions[81,82]. In this context, a commensal bacterium activates the focal adhesion kinase, thereby enhancing epithelial restitution and promoting mucosal wound repair in a redox-dependent manner[83-85]. In a mouse colonoscopy-based wound healing model, an abundance of anaerobic bacteria such as *Akkermansia spp* augmented early stages of MH[86]. The selection of mucin-producing bacteria from the mucin layer also helps close mucosal wounds[87,88]. These microbes help generate SCFAs such as acetate, propionate, and butyrate, which are considered the primary energy sources of gut colonocytes and are therefore critical supporters of IEB restoration and integrity following tissue damage[89-92]. The major producers of SCFAs include the genus *Bacteroides*, *Clostridium* clusters IV and XIVa, and *Bifidobacterium*, though they use diverse mechanisms to achieve homeostatic outcomes[89,93,94]. For example, *Bacteroides ovatus* decreases lipopolysaccharide-induced inflammation and produces indole-3-acetic acid that likely promotes IL-22 production by immune cells, yielding beneficial effects in epithelial regeneration[95,96]. SCFAs produced by fiber-fermenting commensal microbes are also linked to upregulation of Foxp3+ T regulatory (Tregs) cell development, which have a widely documented role in protection against epithelial injury and colitis [97]. Inhibition of histone deacetylases and/or activation of the latent form of transforming growth factor-β (TGF-β) to act as a potent inducer of Tregs are potential mechanisms of SCFAs[98,99]. SCFAs also mediate activation of STAT3 which plays a vital role in mucosal homeostasis[100,101]. *Clostridia*-related segmented filamentous bacteria promote IL-23 production by antigen-presenting cells, which activate type 3 innate lymphoid cells (ILCs) to initiate an IL-23R/IL-22/STAT3 loop, thereby producing serum amyloid A which promotes IL-17 production by Th17 cells[102-105]. The importance of the SCFA propionate is the augmentation of dendritic cell and macrophage hematopoiesis precursors that impact intestinal immunity to control the growth of invading mucosal pathogens[106,107]. A breach in this regulation is a central mechanism in triggering, maintaining, and exacerbating IBD. Supplementation with another important SCFA, butyrate, rescues deficiencies in mitochondrial respiration and increases autophagy in the colonocytes of germ-free mice compared to conventionally raised mice[108]. Due to the critical role in repair of gut dysbiosis, the regulation of specific gut bacteria and SCFAs may therefore possess significant potential for clinical treatment of IBD.

As introduced above, accumulating evidence suggests a causal interdependence between autophagic flux in the intestinal epithelium and gut microbiota colonization. In this regard, CD patients who are homozygous for the ATG16L1^{T300A} gene variant exhibit higher abundance of Enterobacteriaceae, Bacteroidaceae and Fusobacteriaceae in the inflamed ileum compared with patients homozygous for the wild

type ATG16L1 allele[6]. Similar findings have been obtained from mice where expression of the autophagy gene Atg7 is genetically knocked out in the gut epithelium, as these mice display altered microbial composition with enrichment in *Clostridium septum*, *Eubacterium cylindroides*, and *Bacteroides fragilis* compared to wild type mice[109]. ATG16L1^{T300A} variant mice also show changes in fecal microbiota composition compared to wild type mice, displaying an increase in the order Bacteroidales, which is associated with increased Th17 and Th1 cells in the colon and ileum lamina propria without the development of intestinal inflammation[7]. However, Atg5 deficient mice display reduced bacterial diversity, as observed in IBD patients, and contain a low number of the Lachnospiraceae, Ruminococcaceae, and Akkermansia families that control inflammatory responses[110]. Of note, a role of autophagy in regulating intestinal stem cell function and mucosal injury/repair has been demonstrated by several recent studies[111,112]. Taken together, these studies highlight a complex causal integration between host cell autophagy processes and intestinal microbial communities in regulating intestinal homeostasis and injury/repair.

Additionally, infiltrating immune cells such as macrophages and neutrophils responding to gut dysbiosis comprise essential components of intestinal wound healing by altering aberrant physiological parameters of the local microenvironment, such as microbe-associated molecular patterns (MAMPs) and decreased oxygen levels from the formation of reactive oxygen species[86,113-115]. Of note, Toll-like receptors (TLRs) expressed on multiple immune cell lineages induce signaling pathways upon binding by MAMPs and improve outcomes in experimental mouse colitis models *via* the promotion of wound healing[116,117]. Specific microbes in proximity to the wound bed also activate host epithelial proliferative signaling through a formyl peptide receptor pathway[83,84]. Studies employing a mechanical colonic wound method further disclose a protective role for prostaglandin E2 (PGE2) in re-establishment of the IEB through a TLR2/MyD88-dependent manner[118,119]. A follow-up study from the same group shows that in the early repair phase, a TLR2/PGE2 axis is required for barrier establishment; however, PGE2 must then decrease to allow for epithelial proliferation and regeneration [118]. In this context, Jain *et al*[120] elegantly demonstrates that temporal regulation of the bacterial metabolite PGE2/deoxycholate during colonic repair is critical for crypt regeneration[120]. The highly specific and time-dependent switching of microbial colonization and signaling pathways can therefore act to promote MH in a localized manner.

Several therapeutic approaches have been examined by administration of prebiotics or probiotics to regulate the microbiota. For example, butyrate enemas are effective in treating experimental colitis and UC patients[121,122]. Also, p40, a protein produced by *Lactobacillus rhamnosus GG* (LGG), activates host epithelial EGFR signaling and mediates wound healing[123]. Of note, the mechanism of MH promotion by LGG is *via* a positive effect on epithelial barrier maturation by upregulation of Claudin-3[124]. Recently, genetically modified probiotic bacteria–based precision delivery of human EGF also appears to be a promising intervention against mucosal inflammation through crypt-derived MH and barrier restoration[125,126]. Firmicutes, such as *Faecalibacterium prausnitzii* play an essential role in mucosal barrier homeostasis by regulating NF-κB activation and IL-8 production[125,126]. In another study, oral gavage with *Faecalibacterium prausnitzii* during dextran sodium sulfate (DSS) colitis improves outcomes compared to mice treated with DSS alone, likely due to participation of Claudin-1 and Claudin-2[125]. The probiotic mixture known as VSL #3, containing 4 strains of *Lactobacilli*, 3 strains of *Bifidobacteria*, and one strain of *Streptococcus* is effective in preventing pouchitis and in treating UC flareups[127]. This probiotic functions by partially upregulating mucin production and restoring the IEB by stimulating ZO-1 and Occludin expression while decreasing Claudin-2[127]. Taken together, a complex interdependence exists between gut microbiota and MH processes in the promotion of barrier integrity that should be fully explored for its intriguing potential in improving clinical outcomes.

Inflammatory Cytokines in IBD and Mucosal Healing

Due to dysregulation of many cytokines and growth factors in IBD and the regulatory importance of many of these same molecules in MH, we discuss the potential of altering immune signaling and inflammatory cascades in restoring proper intestinal homeostatic balance. Increasing knowledge of the coordination of these pathways will contribute to the development of more effective and targeted therapies to ameliorate disease while preserving essential immune system function.

Major cytokines and growth factors that are considered pro-inflammatory in IBD include IL-1β, interferon-gamma (IFN-γ), TNF-α, and IL-6. During an infection in the gut, IL-1β, TNF-α, and IFN-γ are shown to be produced by inflammatory monocytes, among which IL-1β and TNF-α are associated with increased IEB permeability[2]. TNF-α and IFN-γ are also produced by ILCs and serve to recruit and activate additional inflammatory cells[3]. TNF-α is particularly well-studied and often targeted in the treatment of autoimmune diseases as detailed in our discussion of monoclonal antibody therapies above. Although necessary for innate immune responses against acute pathogens and acute DSS colitis, when produced chronically by T cells TNF-α can be a major contributing factor to the loss of epithelial barrier and development of autoimmune disease[128-130]. IFN-γ has been shown to be regulated during wound healing of skin epithelium by Tregs, where lack of Tregs resulted in increased IFN-γ, accumulation of pro-inflammatory macrophages, and hindered wound healing[131]. Gut microbiota can also impact immune system activation through cytokine signaling. Kuhn *et al*.[132] demonstrates that intraepithelial lymphocytes (IELs) must interact with commensal Bacteroidales order microbes to produce IL-

6 in response to acute *C. rodentium* colitis infection, aiding in repair of the IEB *via* increased Claudin-1 expression[132]. Despite the necessary effects of pro-inflammatory cytokines for immune system response and homeostasis, each also possesses drawbacks. When the location, amount, or duration of cytokine production becomes dysregulated, chronic inflammation and disease can result. IFN-γ, TNF-α, and IL-6 are upregulated after secretion by immune cells in the chronic inflammatory state associated with IBD[3,130,132]. These cytokines are known to increase gut permeability by altering tight junction protein expression[132-134]. In particular, increased IL-6 in the IECs and lamina propria mononuclear cells increases Claudin-2, which promotes intestinal permeability and is known to be upregulated in IBD[134,135]. Interestingly, it is IELs that produce IL-6 in a protective manner during acute infection through the c-Jun N-terminal Kinase pathway, rather than the Mitogen-activated protein kinase/extracellular signal-regulated kinase pathway, suggesting both duration and cell type-specific layers of complexity in the role of IL-6[132,134]. It is likely that similar multi-layer and highly context-specific pathways exist for other inflammatory cytokines as well. Therein lies the challenge of harnessing pro-inflammatory cytokines and signaling pathways in the immune system for MH: Targeting modifications toward the decrease of detrimental chronic effects without impairing their beneficial and homeostatic functions. Continuing in the discussion of IL-6, global reduction of IL-6 expression can decrease chronic inflammation in IBD and enhance MH, but may also increase susceptibility to infection[132,136]. It would therefore be ideal to develop a method of reducing IL-6 production in IECs while leaving expression in IELs intact to avoid increasing the risk of dangerous infections in patients. The potential in fine-tuning inflammatory cytokine expression to promote MH is great, but much work remains to ensure it can be accomplished safely without severe detrimental effects to other functions of the immune system.

Conversely, several other cytokines, growth factors, and cell types function primarily in an anti-inflammatory role in IBD and intestinal homeostasis and offer additional mechanisms to enhance MH by resolving chronic inflammation. Growth factors and cytokines considered to be anti-inflammatory include TGF-β, IL-10, IL-22, and IL-17. TGF-β is a well-studied growth factor that has been demonstrated by Beck *et al*[137]. to play a role in the restitution, or IEC migration, phase of MH, evidenced by the lack of IEC migration and impaired wound healing after TGF-β inhibition in DSS colitis[8,137]. Additionally, TGF-β when produced by a macrophage secretome called SuperMApo aids in removal of apoptotic cells and resolution of inflammation in IBD models[57,138]. Importantly, since this secretome produces TGF-β from a singular cell type, rather than globally, its administration can provide the context- and location-dependent production of beneficial TGF-β while avoiding potential opposing or off-target effects. Macrophages and Th2 cells also produce IL-10, which enhances the proliferation phase of MH, maintains immune tolerance to the many antigens encountered by the IEB, and promotes barrier integrity[139,140]. Similarly, the production of IL-17 by anti-inflammatory Tregs helps with IEC proliferation and blockage of detrimental microbiota in colitis models, providing protection against IBD[141]. One of the major players in protection against IBD is IL-22, which is produced by multiple cell types and promotes MH by more than one mechanism, as reviewed in[142]. Most importantly in IBD, IL-22 is produced by ILCs, CD4+ T cells, and NK cells, demonstrating activation of both the innate and adaptive immune system[143,144]. IL-22 primarily acts on intestinal stem cells (ISCs) and IECs and functions by activating STAT3 signaling, which induces IEC proliferation and therefore MH[101,142,143,145]. Regarding ISCs, IL-22 both protects them from depletion during intestinal inflammation and induces regeneration[101,143,145]. Though some of these cytokines and growth factors can be inflammatory under certain circumstances, in the context of IBD they function in an anti-inflammatory manner and are beneficial in promoting MH. Some cytokines even demonstrate potential to be produced or administered selectively in only beneficial locations or cell types. Therefore, anti-inflammatory cytokines and growth factors present promising options for treatment of IBD through selectively reducing inflammatory signals and inducing or enhancing the MH process.

CONCLUSION

In recent years, IBD has become increasingly common in the United States and abroad. As progressive and debilitating diseases, UC and CD have long-term negative implications on health. In the past, treatments have focused on reducing the clinical manifestations of the disease, often leaving underlying disease mechanisms active in the gut[12,15]. With increased understanding of the mechanisms and complex pathogenesis of IBD, further innovation in treatment approaches must occur to improve patients' long-term outcomes. Subsequently, instead of merely hoping to reduce symptoms, doctors now desire therapies that actively aid in healing and regeneration of damaged tissue. MH has therefore emerged as the main goal for research and treatment end points to ensure long-term remission, survival, and a good quality of life for patients. Going forward, understanding the interactions regulating the breakdown and regeneration of the IEB, as well as overarching gut homeostasis processes, will be paramount to treating and curing patients with IBD. Monoclonal antibody therapies offer a promising start to revolutionizing treatment, aiming not only to reduce clinical manifestations, but also to interrupt disease activity on a cellular and molecular level. However, even newly developed antibody therapies

cannot by themselves completely resolve IBD and restore total gut homeostasis. Therefore, the three approaches to targeting the molecular machinery governing IBD of restoring the IEB, regulating the gut microbiota, and altering the cytokine signaling-mediated immune response are all being studied as potential mechanisms for achieving MH. Optimal future treatment protocols for IBD will ideally include a combination of these approaches, with the intent of restoring intestinal homeostasis by balancing expression of multiple proteins and repairing several of the many dysregulated pathways involved in IBD pathogenesis.

FOOTNOTES

Author contributions: Otte ML, Lama Tamang R, Papapanagiotou J, and Ahmad R reviewed literature for the latest advances in the field; Otte ML, Lama Tamang R, Papapanagiotou J, Ahmad R, Dhawan P, and Singh AB wrote and revised this paper.

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Country/Territory of origin: United States

ORCID number: Megan Lynn Otte 0000-0003-2984-8198; Rizwan Ahmad 0000-0003-0331-6687; Punita Dhawan 0000-0003-3434-8155; Amar B Singh 0000-0003-1908-8594.

Corresponding Author's Membership in Professional Societies: American Gastrointestinal Association (AGA), 992160.

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MINIREVIEWS

Choosing the best endoscopic approach for post-bariatric surgical leaks and fistulas: Basic principles and recommendations

Victor Lira de Oliveira, Alexandre Moraes Bestetti, Roberto Paolo Trasolini, Eduardo Guimarães Hourneaux de Moura, Diogo Turiani Hourneaux de Moura

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Victor Lira de Oliveira, Alexandre Moraes Bestetti, Eduardo Guimarães Hourneaux de Moura, Diogo Turiani Hourneaux de Moura, Serviço de Endoscopia Gastrointestinal, Departamento de Gastroenterologia, Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo 05403010, Brazil

Roberto Paolo Trasolini, Division of Gastroenterology, Hepatology and Endoscopy, Harvard Medical School, Brigham and Women's Hospital, Boston, MA 021115, United States

Corresponding author: Diogo Turiani Hourneaux de Moura, MD, MSc, PhD, Research Fellow, Professor, Serviço de Endoscopia Gastrointestinal, Departamento de Gastroenterologia, Faculdade de Medicina da Universidade de São Paulo, Av. Dr Enéas de Carvalho Aguiar, 255, 6 Andar, Bloco 3, Cerqueira Cesar, São Paulo 05403010, Brazil. dthmoura@hotmail.com

Abstract

Post-surgical leaks and fistulas are the most feared complication of bariatric surgery. They have become more common in clinical practice given the increasing number of these procedures and can be very difficult to treat. These two related conditions must be distinguished and characterized to guide the appropriate treatment. Leak is defined as a transmural defect with communication between the intra and extraluminal compartments, while fistula is defined as an abnormal communication between two epithelialized surfaces. Traditionally, surgical treatment was the preferred approach for leaks and fistulas and was associated with high morbidity with significant mortality rates. However, with the development of novel devices and techniques, endoscopic therapy plays an increasingly essential role in managing these conditions. Early diagnosis and endoscopic therapy initiation after clinical stabilization are crucial to success since clinical success rates are higher for acute leaks and fistulas when compared to late and chronic leaks and fistulas. Several endoscopic techniques are available with different mechanisms of action, including direct closure, covering/diverting or draining. The treatment should be individualized by considering the characteristics of both the patient and the defect. Although there is a lack of high-quality studies to provide standardized treatment algorithms, this narrative review aims to provide a summary of the current scientific evidence and, based on this data and our extensive experience, make recommendations to help choose the best endoscopic approach for the management of post-bariatric surgical leaks and fistulas.

Key Words: Endoscopy; Surgery; Bariatric; Gastrointestinal; Fistulas; Leaks

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Core Tip: Post-surgical leaks and fistulas are the most feared complications of bariatric surgery. Endoscopic therapy is essential for effective management of these conditions. Several endoscopic techniques are available, and this review aims to clarify their mechanisms of action, basic principles, and optimal approach for each situation based on a detailed literature review as well as the authors' personal experience.

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INTRODUCTION

Obesity is now a pandemic, and the prevalence of people living with obesity continues to increase. As a chronic and multifactorial disease with several associated comorbidities, a multidisciplinary approach is required to prevent, treat, and reverse obesity-related complications and to improve quality and length of life for people with obesity[1].

Bariatric and metabolic surgery (BMS) remains the most effective and durable therapy for weight loss and improvement of associated comorbidities. Unsurprisingly, the number of bariatric surgeries performed has grown progressively worldwide. In 2019, about 256000 BMS were performed in the United States. The most performed procedure is laparoscopic sleeve gastrectomy (LSG) followed by Roux-en-Y gastric bypass (RYGB), and revisional surgery[2]. With the increasing number of BMS, familiarity with management of procedure related complications is increasingly important for endoscopists.

Although rare, especially in referral centers, complications can occur, such as leaks and fistulas[3]. Traditionally, leaks and fistulas were treated with surgery. However, surgical management of these defects is usually challenging and has been associated with high morbidity and mortality rates[4]. Less invasive approaches are preferred, when possible, to reduce associated morbidity. Endoscopic therapies play an essential role in the management of leaks and fistulas. Several endoscopic devices and techniques, with different mechanisms of action have been developed, with considerable clinical success for what is often morbid and refractory disease[3,4].

In this narrative review we discuss the pathophysiology, characteristics, diagnosis, and management of post-bariatric surgical leaks and fistulas, focusing on endoscopic therapies. We include descriptions of the mechanism of action, indications, contraindications, tips, tricks, and outcomes for different techniques, in order to facilitate choosing the best approach for each unique case.

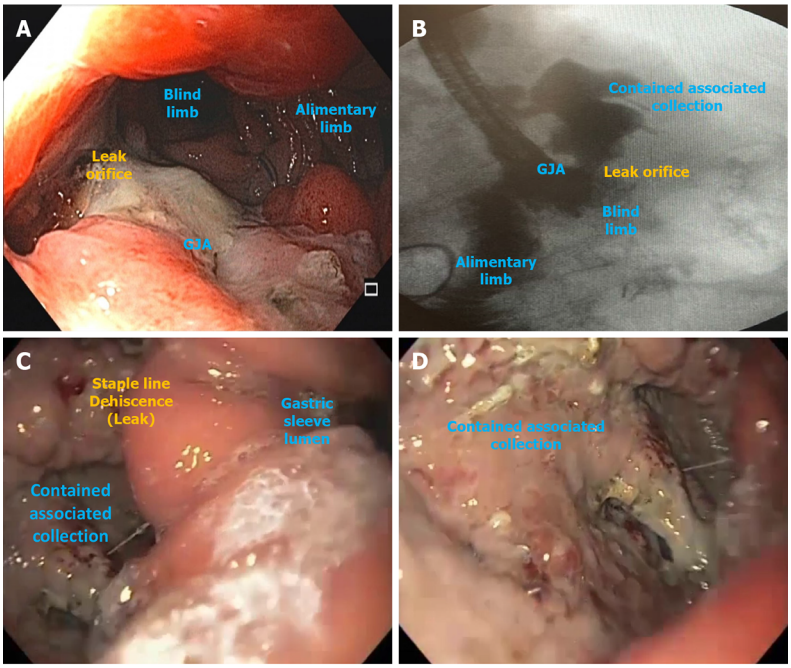
DEFINITIONS

Leak is defined as a transmural defect with communication between the intra and extraluminal compartments (Figure 1). A fistula is defined as an abnormal communication between two epithelialized surfaces. Fistulas can be divided into internal and external. An internal fistula occurs between two internal epithelialized organs, whereas an external fistula is a communication between an internal organ and the skin surface (Figure 2)[5].

EPIDEMIOLOGY

Despite the increasing number of BMS, the rate of mortality and adverse events (AEs), including leaks and fistulas, has decreased over the past two decades[6] due to improvement in surgical techniques, such as laparoscopy and robotic surgery, improved materials, and surgeons' expertise.

Leaks and fistulas occurred with similar incidence among the most common bariatric surgeries, ranging from 0.4% to 5.6% in RYGB and 1.9% to 5.3% in LSG, with higher rates after revisional surgeries



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Figure 1 Post-bariatric surgical leaks illustrations. A and B: Gastrojejunal anastomotic leak with an associated contained collection after Roux-en-Y gastric bypass; C and D: Leak with a contained associated collection due to staple line dehiscence after laparoscopic sleeve gastrectomy. GJA: Gastrojejunal anastomosis.

[7-10]. This incidence is considerably lower in high-volume specialized centers; as low as 0.5%[11]. Post-BMS mortality is very low, ranging from 0.2% to 0.4%, and is mainly related to late diagnosis and management of complications[7-10].

CAUSES AND PREVENTION

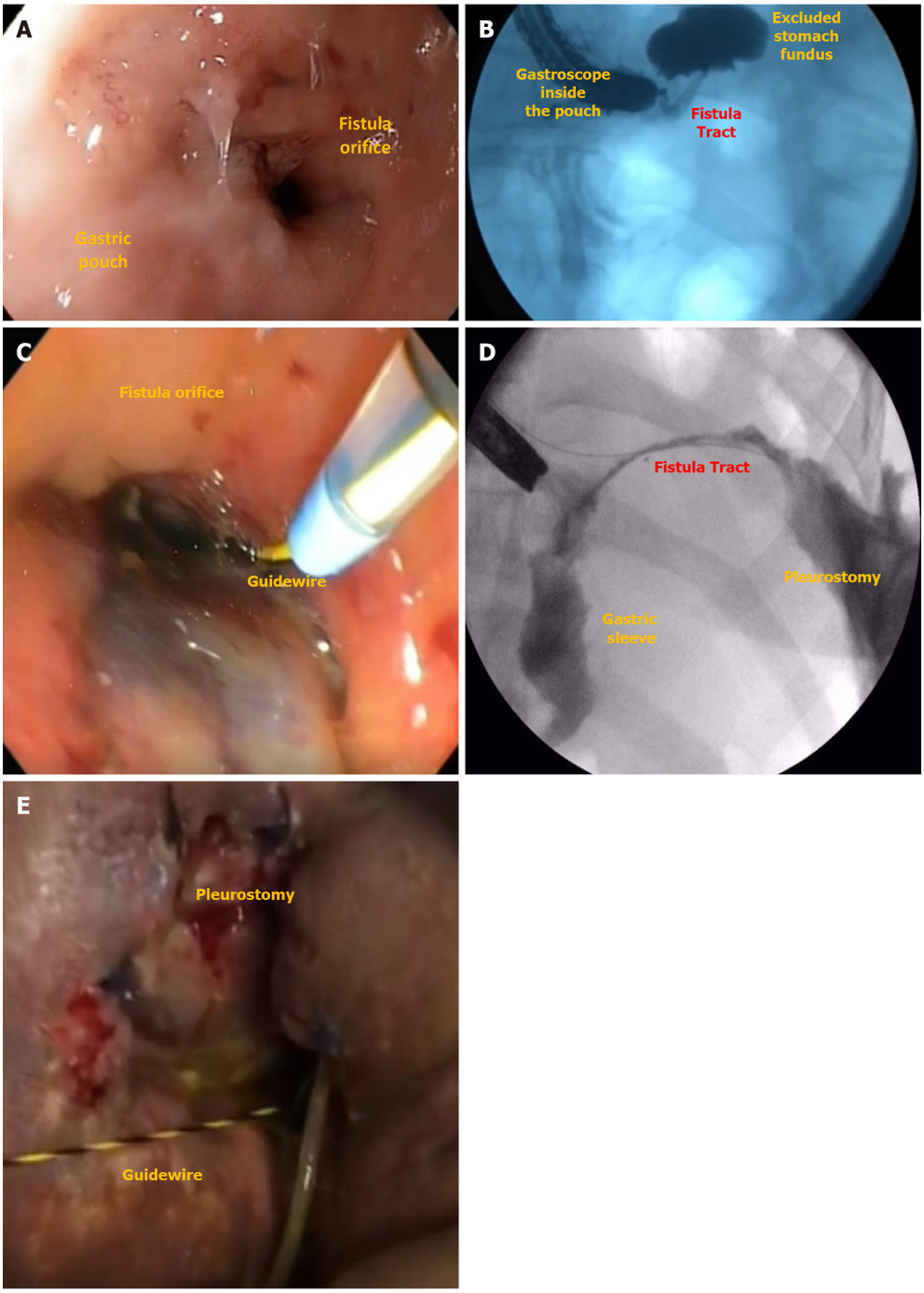
Leaks usually occur after surgery and are often located at the suture (staple) line and/or anastomosis, while fistulas are mainly caused by untreated leaks[5].

The causes of transmural defects after BMS are related to several factors, including the patient related risk factors and technical risk factors. Patient related risk factors for transmural defects include high body mass index, age, smoking, alcohol use, malnutrition, and other related comorbidities [*e.g.*, type 2 diabetes mellitus (T2DM)][12]. Technical risk factors include mechanical or ischemic insults, both of which contribute to a mechanism of high intraluminal pressure that overcomes the integrity of the tissue. Meticulous surgical technique, with careful attention to tissue handling and inadvertent excessive tension on anastomoses, luminal narrowing, suturing/stapling ischemic areas, and torsion of blood vessels or organs is mandatory to avoid undesirable outcomes such as downstream stenosis, leaks, and fistulas. Additionally, stapling failure is also a cause of BMS leaks[13,14].

The pathophysiology of leaks and fistulas after RYGB and LSG differ; given distinct technical and anatomic particularities of each surgery. Most commonly in LSG, the creation of a long and narrow conduit combined with pylorus preservation creates a high-pressure region proximally leading to stress on the proximal staple line. In RYGB, the gastric pouch is a low-pressure system with low resistance to gastric emptying through the gastrojejunal anastomosis (GJA) into the small bowel, though high-pressure areas can develop in cases of GJA stenosis or excluded stomach gastroparesis. The anatomy of LSG favors leak and fistula formation at the proximal portion of the staple line, especially near the angle of His. Here the thin gastric wall, high pressure, and borderline vascularization secondary to take down of the short gastric arteries all contribute to comprised wall integrity[14-16].

Of note, for both LSG and RYGB, it has been shown that laparoscopic surgery reduces the incidence of leaks and fistulas as well as mortality rates compared to open surgery[6,17]. Intraoperative leak assessment using methylene blue, endoscopy, or air insufflation allows for immediate repair of defects though this practice has never been proven to reduce the incidence of leak or fistula formation after surgery[14,18].

Stapling technology has evolved in recent years with the development of different staple heights capable of accommodating variable gastric wall thickness according to the anatomic portions of the stomach. To take advantage of this technology, the surgeon must be aware of which one is the most appropriate in each case to avoid mismatch[14]. Moreover, several techniques of staple line reinforcement have been studied, from oversewing the staple line with different suture techniques to



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Figure 2 Post-bariatric surgical fistulas illustrations. A and B: Gastrogastric fistula after Roux-en-Y gastric bypass; C, D, and E: Gastropleural fistula after laparoscopic sleeve gastrectomy.

the use of biologic or synthetic materials, such as glues and tissue sealants, but none of these interventions have been proven to reduce the incidence of post-surgical leaks and fistulas[19-22].

The routine placement of drains near the anastomosis is controversial and there is no consensus in the literature and between surgeons on whether this intervention prevents the development of uncontrolled leakage or increases the risk of developing leaks[23,24].

CLASSIFICATION

Leaks and fistulas can be classified based on several parameters, including time of onset and location.

The definition of acute *vs* chronic leaks and fistulas varies between 30 and 45 d in the literature (acute < 30-45 d and chronic > 30-45 d)[25,26]. In our practice, we use the classification described in the

international LSG consensus, which classified the defects into acute (< 7 d), early (between 7 d and 6 wk or 45 d), late (between 6 wk or 45 d and 3 mo or 90 d), and chronic (> 3 mo or 90 d)[27]. Up to 20% of acute and early leaks and fistulas become late and chronic leaks and fistulas, mainly when the defect is untreated[26,28].

Based on the location, post-RYGB leaks are classified into Type I to VI. The locations include: The gastric pouch (Type I), GJA (Type II), blind jejunal stump (Type III), jejunojejunal anastomosis (Type IV), excluded stomach (Type V), and the blind stump of the biliopancreatic limb (Type VI). The first three topographies are easily accessible by endoscopy and can be treated with endoscopic techniques, unlike the others[29].

LSG leaks mostly occur at the level of the angle of His due to poor vascularization (more than 90% of the cases) but can also occur in the distal body and antrum. Both locations can be treated with an endoscopic approach[27].

DIAGNOSIS

The diagnosis of leaks and fistulas is based on clinical history and physical examination. Complementary studies, including laboratory tests and imaging are usually required to establish a precise diagnosis.

Clinical manifestations vary according to individual factors related to the level of inflammatory response as well as particularities of the leak or fistula, such as the onset time, defect location, and the placement of a post-surgical drain. If an external drain is placed, the leak can be identified early due to increased drainage[5]. On the other hand, patients without external drainage usually present with infectious manifestations, from mild signs and symptoms, such as low-grade tachycardia, fever, and abdominal pain, to sepsis[30].

Clinical manifestations of fistulas depend on the location of the defect. Respiratory symptoms should be an alarm to consider gastrointestinal (GI)-respiratory fistulas, although patients with peritonitis can also present with pleural effusion[31]. GI-cutaneous fistulas are easily diagnosed if secretions are observed coming out through the skin[32]. Gastrogastric fistula is usually a late complication of RYGB and should be investigated in patients with weight regain and/or gastroesophageal reflux[33].

Laboratory tests are important in acute infection and include leukocytosis, elevated C-reactive protein, and changes indicating organ dysfunction[14]. Additionally, it may be important in patients with long-term fistulas to evaluate nutritional state or electrolyte depletion, for example, in chronic gastrocolic fistulas.

Once suspicion is established, diagnosis is usually confirmed with imaging exams, including radiography of the abdomen, upper gastro-intestinal transit (upper GI series) and computed tomography (CT) scan with administration of water-soluble oral contrast, esophagogastroduodenoscopy (EGD), and/or fistulogram[34] (Figure 3).

Radiography of the abdomen is frequently requested as it can rapidly evaluate for pneumoperitoneum (Figure 3A).

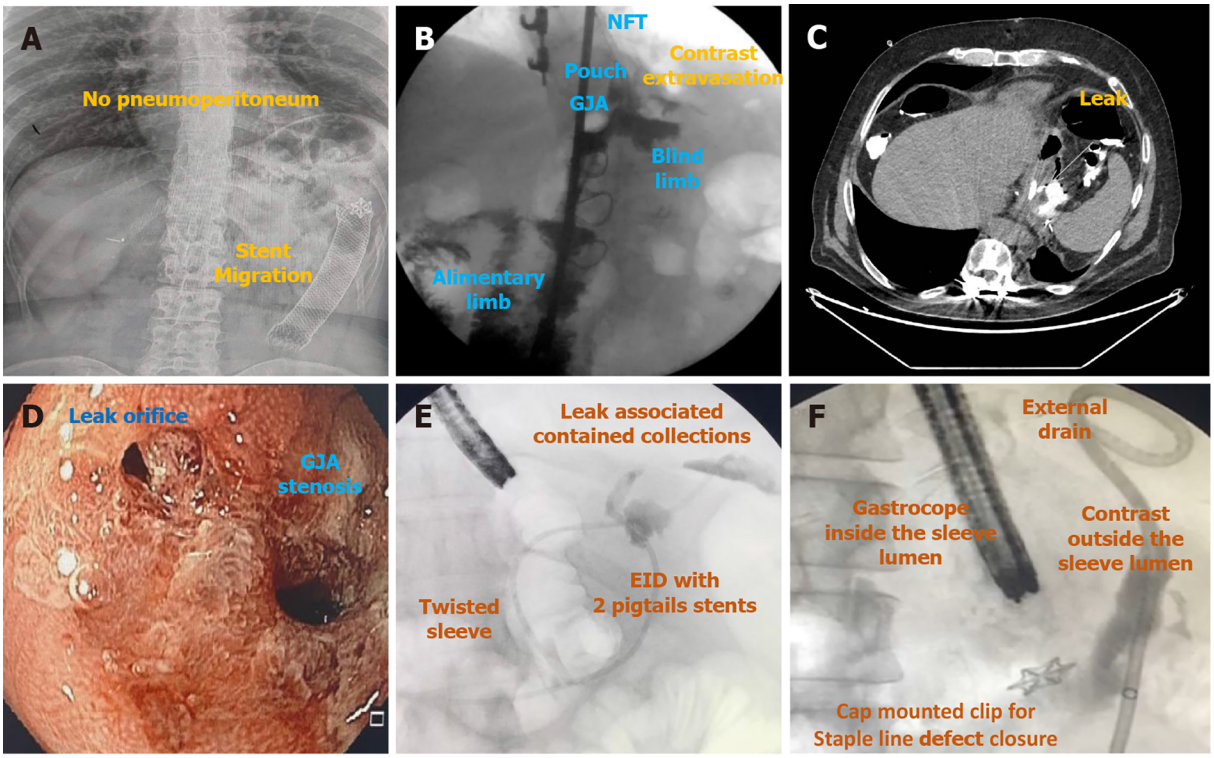
Upper GI transit should be performed with water-soluble contrast if leak or fistula are suspected (Figure 3B). It is important to locate the fistulous orifice and to understand the post-surgical anatomy, including the presence of downstream stenosis for successful treatment. The use of barium is not recommended as it may delay endoscopic treatment. When GI transit findings are doubtful or clinically discordant, CT scan should be used to clarify the results due to its lower sensitivity[35,36].

CT scan allows the evaluation of a fistulous path as well as indirect signs of the source of a leak or fistula, presence of associated collections, pneumoperitoneum, and free fluid. Additionally, it provides a broad evaluation of the intracavitary organs for procedural planning (Figure 3C)[4,37].

EGD is needed to evaluate the fistulous orifice (Figure 3D) surrounding tissue, presence of foreign bodies, and downstream stenosis. Fluoroscopy can be critically useful, especially for orifices smaller than the diameter of the gastroscope. In these cases, a fistulogram can be performed by injecting water-soluble contrast through a catheter or the gastroscope working channel (Figure 3E). Additionally, injection of water-soluble contrast, methylene blue, or a bubble test can be performed if there is an external drain (Figure 3F)[5]. In patients with LSG and suspicion of a leak or fistula without an evident defect, an important reminder is to follow the staple line superiorly looking for small orifices as these can be difficult to locate. A fistulogram can also be performed percutaneously in cases of GI-cutaneous fistulas allowing for a better understanding (size and location) of the fistula tract.

PRINCIPLES OF TREATMENT

The treatment of leaks and fistulas is based on four pivotal principles: (1) Clinical management; (2) Drainage; (3) Treatment of associated factors; and (4) Closure of the transmural defect.



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Figure 3 Imaging exams for diagnosis of leaks and fistulas after bariatric surgery. A: Abdominal radiograph showing an esophageal fully covered metal stent fixed with a cap mounted clip at its proximal edge used for the treatment of a gastrojejunal anastomosis (GJA) leak after Roux-en-Y gastric bypass (RYGB) migrated to distal jejunum with no signs of pneuperitoneum; B: Upper gastrointestinal (GI) transit for suspicious leak after RYGB showing contrast extravasation; C: Computed tomography scan confirming the GJA leak suspected in the upper GI series (Figure 3B); D: Endoscopic visualization of a GJA stenosis associated with a leak; E: Fluoroscopy image during esophagogastroduodenoscopy (EGD) with injection of water soluble contrast through a catheter showing two leaks in the sleeve staple line with associated collections treated with endoscopic internal drainage with pigtail stents; F: Fluoroscopy image during EGD with injection of water soluble contrast through the external drain showing no extravasation for the intraluminal compartment, confirming the successful defect closure. GJA: Gastrojejunal anastomosis; EID: Endoscopic internal drainage; NFT: Nasoenteral feeding tube.

First, unstable patients must be clinically stabilized, including fluid resuscitation, packed blood cells transfusion when indicated, and administration of vasoactive drugs if necessary.

Infectious source control including intravenous antibiotic therapy and drainage must be performed to control sepsis. Drainage can be performed surgically, percutaneously by image guidance, or endoscopically. For unstable patients with peritonitis and free infected fluid in the cavity, surgical lavage and drainage, with or without defect repair, is the preferred approach.

Nutrition is also key for defect healing and is often neglected as most patients are kept nil *per* os initially. Therefore, enteral or parenteral nutrition should be introduced as early as feasible. Enteral nutrition is the preferred option, through a nasoenteral feeding tube placed distal to the defect[5].

Defect related factors must be treated to achieve successful closure, such as dilation of downstream stenosis, and foreign body removal (staples, suture, and drains, *etc.*)[38,39].

After clinical stabilization, endoscopic evaluation is recommended. The endoscopist must communicate with other specialty teams and review the operative report thoroughly before performing the initial endoscopic evaluation. The role of endoscopy in the management of leaks and fistulas includes both diagnosis and treatment. Early diagnosis and endoscopic management are key for success, with closure efficacy as high as 90% when defects are treated within 3 wk of surgery and about 70% after this period[25,26]. Endoscopic treatment of leaks is associated with higher rates of successful closure when compared to fistulas[40]. Despite the lower efficacy of endoscopic treatment for chronic leaks and fistulas compared to acute and early transmural defects, it should be attempted exhaustively before referral for definitive surgical treatment as surgery is often challenging and associated with more morbidity[41]. In this setting, the most commonly proposed revision surgeries are fistulojejunostomy, conversion of LSG to RYGB (without gastrectomy), and total/near total gastrectomy with esophagojejunal anastomosis. Direct surgical repair of the fistula site is not effective and not advised.

ENDOSCOPIC MANAGEMENT

Endoscopic evaluation of leaks must be performed under carbon dioxide insufflation or underwater to reduce the risk of pneumoperitoneum or pneumomediastinum, especially when there is no external drainage. Careful endoscopic assessment is essential to avoid wall rupture of a contained collection[37].

The procedure can be performed in the operating room, endoscopy suite, or at the bedside[4]. The endoscopy suite is preferable due to its lower costs when compared to the operating room and greater resources when compared to the bedside. However, for unstable patients, the operating room or at bedside in an intensive care unit may be preferred.

General anesthesia with orotracheal intubation to reduce the risk of aspiration during fluoroscopy is advised, especially in the initial evaluation. General anesthesia often allows a more detailed inspection of the transmural defect, including defect size, fistula path, and presence of extraluminal collection[4]. The need for general anesthesia and fluoroscopy is not mandatory but can be tailored to the case at hand.

ENDOSCOPIC TECHNIQUES

Endoscopic therapies utilize several mechanisms of action and can be classified into closure, covering, and draining techniques[5].

Closure techniques include clips [through-the-scope clips (TTSC) and cap mounted clips], endoloop, endoscopic suture, and tissue sealants/glues. Despite some studies[42,43] reporting successful closure of leaks and fistulas using the widely available and simple-to-place TTSC, sometimes combined with endoloop, this technique requires robust and healthy tissue around the defect for successful primary closure. Therefore, this approach is not effective in closing leaks and fistulas and should not be recommended[5]. Hence, this closure technique will not be discussed in this review. Common closure techniques are summarized in Table 1.

Covering techniques utilize cardiac septal defect occluders (CSDO) or self-expandable metal stents (SEMS), including conventional (esophageal) stents and custom (bariatric) stents (Table 2). Closure and covering techniques do not allow internal drainage; thus, external drainage is required when an associated collection or intracavitary infected fluid is identified.

Endoscopic drainage techniques include endoscopic vacuum therapy (EVT), endoscopic internal drainage (EID) with double pigtail stent (DPS), and septotomy (Table 3).

All endoscopic techniques are summarized in Tables 1, 2, and 3 discussed in detail below.

Cap-mounted clips

Cap-mounted clips (Figure 4) are usually referred to as over the scope clips (OTSC®, Ovesco Endoscopy GmbH, Tübingen, Germany) as this is the commercial name of the first cap mounted clip. However, it is important to state that the name OTSC® is trademarked and other devices are available.

Cap mounted clip use is increasing due to its benefits compared to the TTSC, including the ability to close leaks and fistulas as it can approximate a larger volume of tissue.

The device is placed into the distal tip of the endoscope and deployment is similar to band ligation, a procedure widely performed by endoscopists, helping to shorten the learning curve[44]. It can only be used in defects smaller than 20 mm.

A meta-analysis including 73 patients from 9 studies evaluating cap-mounted clips for treatment of leaks and fistulas after LSG, including both acute and chronic defects showed a clinical success rate of 63.5% when used as a single therapy and 86.3% when combined with other therapies[45].

In our experience, cap-mounted clips can be used in patients with small orifices without undrained associated collection and always combine with other therapy (Figure 4A). Additionally, cap-mounted clips can be used for stent fixation (Figure 4B and C).

Tissue sealants/Glues

Tissue sealants (Figure 5A) and glues (Figure 5B) include fibrin glue, acellular matrix biomaterial (SurgiSIS® – Cook Medical, Winston-Salem, North Carolina, United States), and cyanoacrylate. These materials are primarily used to close fistulous tracts and are usually used as an adjunctive therapy.

Despite the closure effect of both glues and tissue sealants, the last group also play a role in tissue healing. Fibrin glue induces a cellular response, extracellular matrix formation and neovascularization, while acellular matrix biomaterial induces fibroblast proliferation[46,47].

Although these techniques have a well-established safety profile, reported clinical success is variable and multiple applications are usually required. In a recent meta-analysis evaluating 10 case series involving 63 patients, the number of sessions needed for treatment ranged from 1 to 9, with a pooled successful closure rate of 96.8%[48]. Regardless of the excellent result shown in this meta-analysis, our experience does not reflect this high efficacy. It is important to analyze this data with care given significant limitations and risk of bias for retrospective case series. Furthermore, clinical success is related to the characteristics of the leak or fistula, including defect site, chronicity, and size. Therefore,

Table 1 Endoscopic closure techniques

Endoscopic techniques	Indications/advantages	Not indicated/disadvantages	Authors experience
Cap-mounted clips	(1) Acute/ early/ late/ chronic; (2) Small orifices (< 20 mm); and (3) Safe	(1) Orifices > 20 mm; (2) Need for external drainage; and (3) Variable efficacy	(1) Acute/ early/ late/ chronic; (2) Safe; (3) < 10 mm: > efficacy; (4) > 10mm: very low efficacy; (5) Combined therapy improves its efficacy; and (6) Can be removed when fails to close the defect (not easy to remove)
Glues/ tissue sealants	(1) Acute/ early/ late/ chronic; (2) Diameter < 10 mm; (3) Low drainage (< 200 ml/24 h); and (4) Safe	(1) Multiple sessions are usually required; (2) Need for external drainage; and (3) Variable efficacy	(1) Late/ chronic; (2) Low efficacy; (3) Safe; (4) Helpful as an adjunctive therapy; (5) Never use it as a single therapy; (6) Multiple sessions; (7) Cytology brushing or APC is useful to loosen granulation tissue before application; (8) Delivery *via* endoscopic or percutaneous access; and (9) High cost (tissue sealants)
Endoscopic suturing	(1) Acute/ early/ late/ chronic; (2) High technical success; and (3) Safe	(1) Need for external drainage; (2) Low efficacy (need for robust and healthy tissue for primary closure); (3) Challenging: Previous experience with the device is needed; and (4) High costs (most countries)	(1) Very poor long-term clinical success; (2) Helpful for other devices fixation; (3) Not recommended for chronic defects; and (4) High cost

APC: Argon plasma coagulation.

Table 2 Endoscopic covering techniques

Endoscopic techniques	Indications/advantages	Not indicated/disadvantages	Authors experience
Conventional (esophageal) stents	(1) Acute/ early; (2) Satisfactory efficacy; (3) Very popular; (4) Widely available; (5) International guidelines support; (6) Easy placement; (7) Early oral intake; and (8) Low number of repeated procedures	(1) Late/ chronic; (2) High migration rates; (3) Need for external drainage; (4) Mild symptoms related to the stent; and (5) Possible "surprise" when removing it	(1) Acute/ early; (2) Satisfactory efficacy; (3) Easy placement/ not expensive; (4) Helpful for complete dehiscence; (5) PCSEMS > FCSEMS (challenging removal – do not keep it for > 3 wk); and (6) High migration rates (FCSEMS)
Bariatric stents	(1) Acute/Early; (2) Satisfactory efficacy; (3) "Perfect" shape for LSG leaks; (4) Low number of repeated procedures ; and (5) Easy placement	(1) Late/ chronic; (2) High migration rates; (3) Need for external drainage; (4) Severe symptoms related to the stent; and (5) Possible "surprise" when removing it	(1) Acute/ early; (2) Similar efficacy to the conventional stent; (3) More expensive than the conventional stent; (4) Helpful for downstream stenosis and complete dehiscence; (5) Pre-pyloric position: more symptoms; (6) Post-pyloric position: more migration; (7) High rates of adverse events (ulcers and perforations); and (8) Intolerance due to symptoms related to the stent (GERD, pain, and emesis)
Cardiac septal defect occluder	(1) Late/ chronic; (2) High efficacy; (3) Safe; and (4) Epithelialized surface is required for device fixation	(1) Need for external drainage; (2) Off-label use; (3) High cost; and (4) Acute and early: enlargement and migration if no epithelialized surface	(1) Very high efficacy for late/ chronic defects with epithelialized tract without associated collection; (2) Safe; (3) High cost; (4) Off-label; (5) Indicated after conventional techniques failure; and (6) Size selection based on defect size (2:1)

FCSEMS: Fully covered self-expandable metal stents; GERD: Gastroesophageal reflux disease; LSG: Laparoscopic sleeve gastrectomy; PCSEMS: Partially covered self-expandable metal stents.

all these variables should be considered in a treatment plan.

In our experience, the best indication for tissue sealants and glues are GI-cutaneous fistulas, with thin tract and low output and always combined with additional therapies[31]. Tissue sealants and glues can be delivered *via* endoscopic or percutaneous tract, facilitating the procedure[49]. Cytology brushing or ablation of the epithelialized (chronic) tract to induce granulation is recommended.

Endoscopic suturing

Endoscopic suturing (Figure 6) allows for full-thickness closure. However, similar to TTSC, any type of suturing (surgical or endoscopic) needs robust and healthy tissue around the defect for primary closure. Therefore, this technique is not effective in closing most leaks and fistulas and should not be recommended[5]. In a multicenter study evaluating fistula closure by endoscopic suturing, 56 patients underwent the procedure with 100% technical success. Despite the high technical success, only 22.4% of patients achieved clinical success at one year follow-up[50]. Additionally, endoscopic suturing is expensive in most countries and requires specialized training.

Table 3 Endoscopic draining techniques

Endoscopic techniques	Indications/advantages	Not indicated/disadvantages	Authors experience
Endoscopic vacuum therapy	(1) Acute/ early/ late/ chronic; (2) High efficacy in leaks and fistulas with or without associated collection; (3) No need for external drainage; (4) Superior to stent in upper GI tract; and (5) Unique mechanism of action: macro-deformation/ micro-deformation, changes in perfusion/ angiogenesis/exudate control/bacterial clearance	(1) Inability to achieve negative pressure; (2) No endoscopic access; (3) Patient discomfort related to nasogastric tube; (4) Usually repeated procedures are needed (especially when traditional sponge is used); and (5) Longer hospital stay/ high costs (?)	(1) Acute/ early/ late/ chronic; (2) Very high clinical success rates; (3) You must place the EVT system in intracavitary position when an associated collection is identified; (4) Placement of both intracavitary and intraluminal EVT appears to be the best approach; (5) Traditional sponge: challenging placement and removal (mouth), prolonged procedures, and need for multiple exchanges (6) Low-cost modified EVT: easy placement and removal, reduction in procedure time, longer interval between EVT system exchanges, low cost, and low AEs rates; and (7) Modified triple-lumen EVT: drainage and nutrition with one tube through the nares
Endoscopic internal drainage with double pigtail stent	(1) Acute/early/late/chronic; (2) High efficacy; (3) No need for external drainage; (4) Need of an associated collection; (4) Easy placement (7fr – gastroscope); (5) Small or large orifices; and (6) Short hospital stay	(1) Defects without an associated collection; (2) No place to accommodate the DPS (small collection: < 2 cm); (3) Long period for complete healing; (4) Risk of migration, perforation and bleeding; and (5) Usually, fluoroscopy is needed	(1) Acute/ early/ late/ chronic; (2) High clinical success rates; (3) Small orifices with associated collection; (4) Easy placement; (5) Shorter hospital stay/ electives procedures for DPS exchanges; (6) Faster oral intake (start with clear liquids); (7) Better patient acceptance – no symptoms; (8) Long period for complete healing; and (9) Ureteral stents appear to be safer with similar efficacy
Septotomy	(1) Early/late/chronic (> 15 d); (2) High efficacy; (3) Safe; (4) Septum between the orifice/ collection and the gastric lumen; and (5) Must do it when a septum is identified	It is only performed when a septum is identified	(1) Early/late/chronic (> 15 d); (2) Very high clinical success; (3) Usually more than 1 session is required; (4) Cut until the staple line; (5) APC or Knife (APC < bleeding); (6) Always dilate after septotomy; (7) Outpatient procedure; and (8) Septum is the cause of most late/chronic refractory defects treated in a center without experience

APC: Argon plasma coagulation; DPS: Double-pigtail stents; EVT: Endoscopic vacuum therapy; GI: Gastrointestinal.

In our experience, we only use endoscopic suturing as an adjunctive therapy, for example to fix a stent aiming to reduce the risk of migration.

Self-expandable metal stents

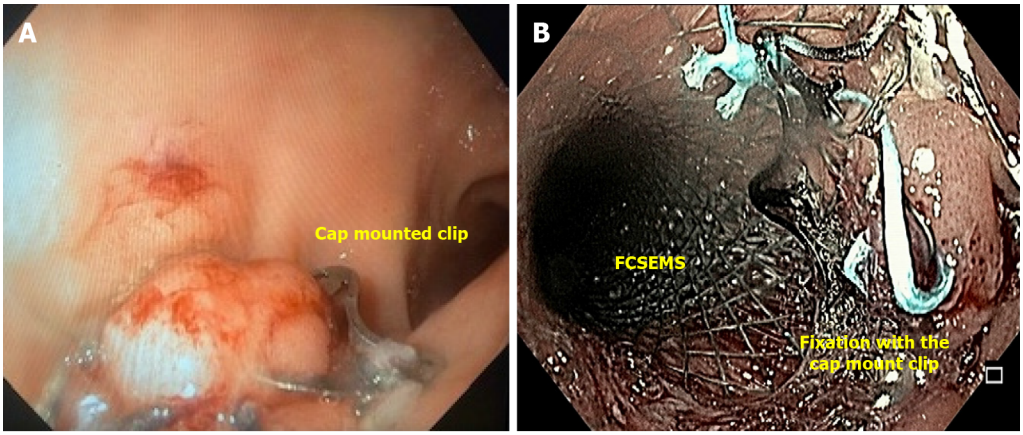
SEMS (Figure 7) are the most used technique for the treatment of post-bariatric surgical leaks and fistulas worldwide and is recommended by the American Society for Metabolic and Bariatric Surgery in a position statement from 2015[14]. SEMS are widely available, easy to place and usually with a low cost.

SEMS are indicated for acute and early leaks and should be avoided in late and chronic defects and late stenosis due to reduced efficacy and increased AE rates, especially migration[51]. The American Gastroenterology Association suggests the use of SEMS for leaks and fistulas earlier than 6 wk, preferably in defects < 10 mm[15].

Despite satisfactory efficacy in acute and early leaks as shown in a recent meta-analysis (RYGB: 76.1% and LSG: 72.8%), high migration rates (RYGB: 30.5% and LSG: 28.2%) must be considered, as an urgent surgery due to device migration may be catastrophic[52]. It is important to remember that SEMS placement requires drainage of any associated collections before or promptly after stent placement.

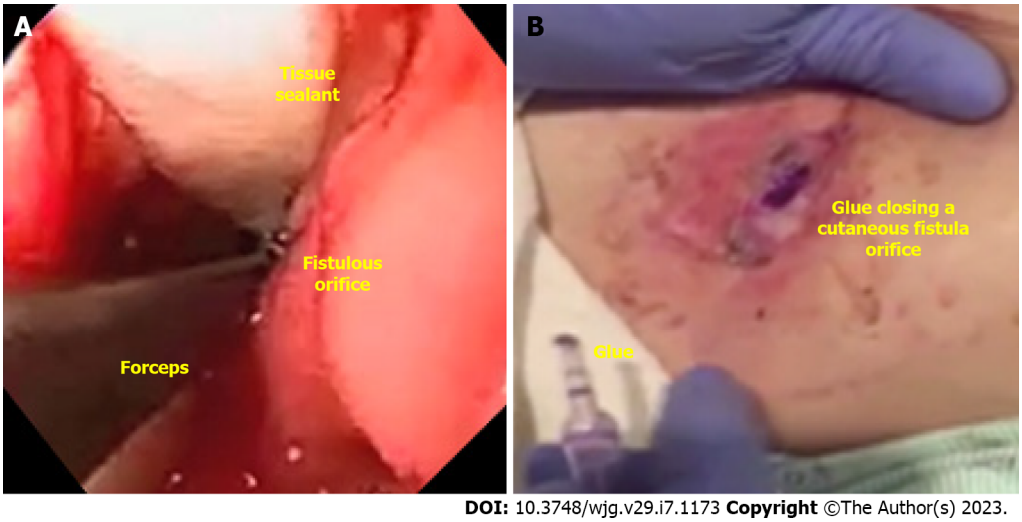
Choosing the correct SEMS facilitates successful therapy. Fully-covered SEMS (FCSEMS) are easy to remove but have a high rate of migration (Figure 7A). Fixation of the FCSEMS with suturing, cap mounted clips or the nasal bridle technique is recommended[53]. On the other hand, partially-covered SEMS (PCSEMS) are associated with low rates of migration, with challenging removal, particularly after 3 wk due to tissue ingrowth (Figure 7B). Nevertheless, some groups argue that the PCSEMS may be better than the FCSEMS as tissue ingrowth prevents leakage between the esophageal wall and the stent. In cases of stent removal failure, several rescue techniques may be used such as the stent in stent technique, ablation and/or endoscopic resection of the tissue ingrowth[15].

Recently, due to the non-negligible migration rate of the esophageal SEMS (Figure 7C), novel models of stents (longer and with a larger diameter) have been developed, focused on the gastric sleeve anatomy. These are customized bariatric stents (Figure 7D). In a retrospective multicenter study[26], 37 patients with early and acute post-LSG leaks were treated with the 24-cm length customized bariatric stent showing similar efficacy and migration rates reported with conventional esophageal SEMS[52]. However, more severe AEs were reported, including two urgent surgeries due to stent migration[54], esophageal perforation[55], contained gastric perforation, and symptoms such as pain and gastroesophageal reflux. In this study, post-pyloric position was associated with high rates of migration and pre-



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Figure 4 Cap-mounted clip. A: Cap-mounted clip for an acute leak (orifice < 2 cm) in the staple line after laparoscopic sleeve gastrectomy in a stable patient with external drainage; B: Cap-mounted clip used for stent fixation; C: Enteroscopy to remove stent after migration due to cap mounted clip fixation failure. FCSEMS: Fully covered self-expandable metal stents.

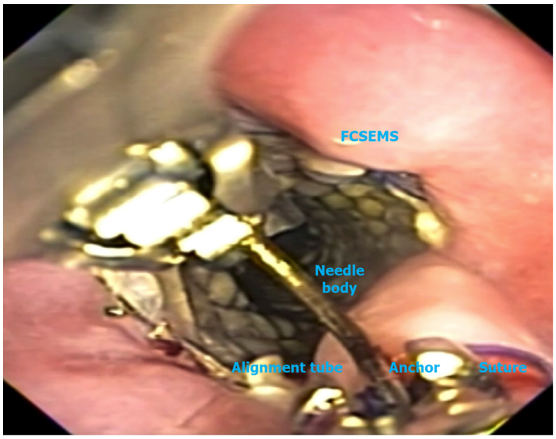


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Figure 5 Tissue sealants/Glues. A: Endoscopic placement of acellular matrix biomaterial in a late gastrocutaneous fistula after laparoscopic sleeve gastrectomy (LSG); B: Percutaneous application of glue (cyanoacrylate) in a chronic gastrocutaneous fistula tract after LSG.

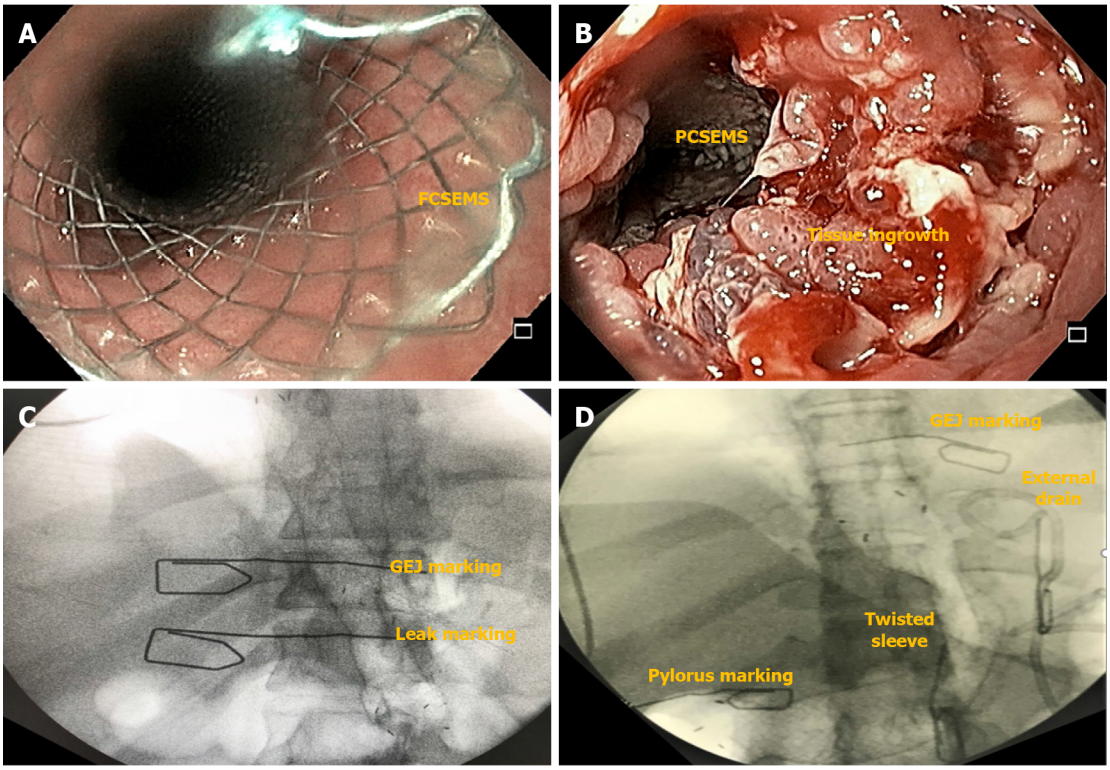
pyloric position with more stent related symptoms[26]. In a meta-analysis comparing conventional esophageal SEMS and the customized bariatric stents, there were no statistical difference in terms of efficacy (93% *vs* 82%) and migration (15% *vs* 32%) rates[56]. Therefore, this novel customized bariatric stent should be used with caution, preferably in specialized centers with close follow up[57].

In our experience, SEMS are best applied in acute and early leaks with complete dehiscence and/or with associated downstream stenosis, always in conjunction with external drainage. Moreover, we



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Figure 6 Endoscopic suturing for stent fixation placed in the distal esophagus of a patient with an early leak after Roux-en-Y gastric bypass. FCSEMS: Fully covered self-expandable metal stents.



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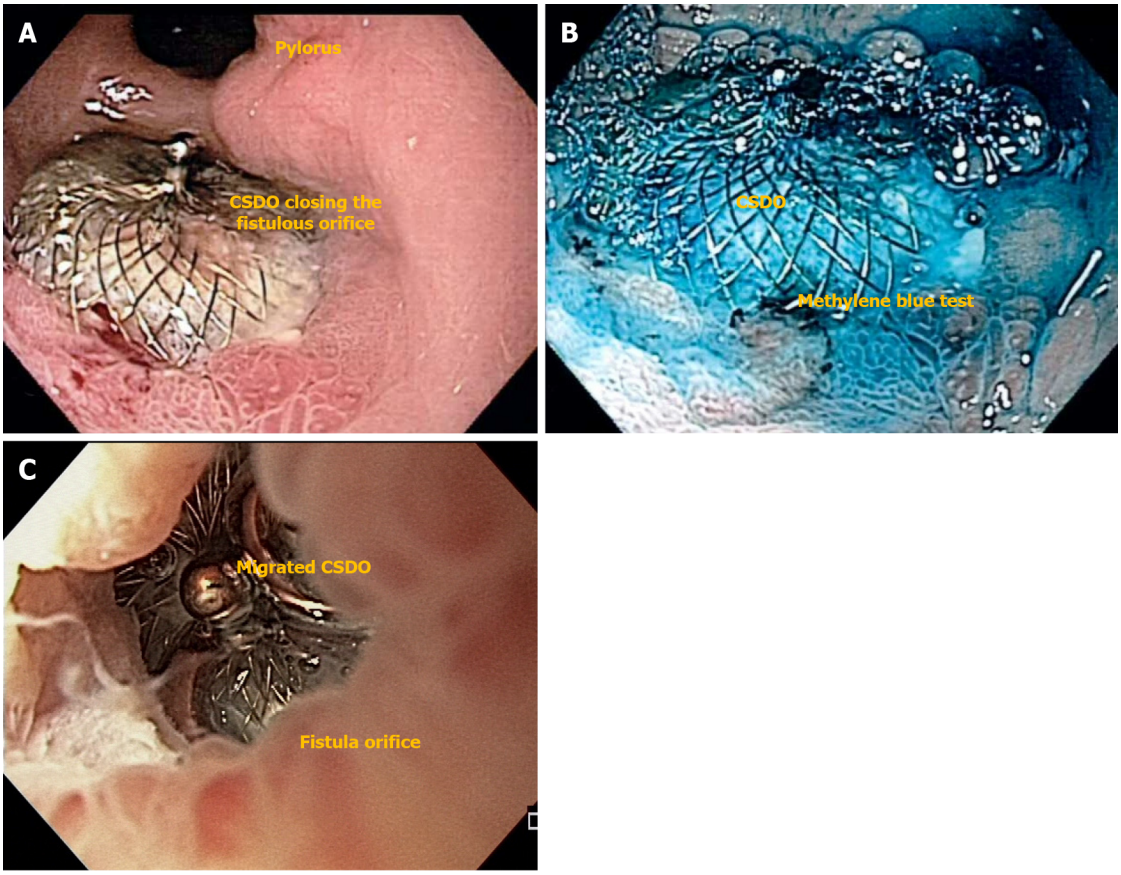
Figure 7 Stents for the treatment of post bariatric and metabolic surgery leaks. A: Esophageal fully covered self-expandable metal stent; B: Tissue ingrowth in an esophageal partially covered self-expandable metal stent; C: Esophageal stent for the treatment of an acute leak after Roux-en-Y gastric bypass; D: Customized bariatric stent for the management of an acute leak in the angle of His after laparoscopic sleeve gastrectomy showing a stenosis at the level of the incisura angularis. FCSEMS: Fully covered self-expandable metal stents; GEJ: Gastroesophageal junction; PCSEMS: Partially covered self-expandable metal stents.

prefer the esophageal PCSEMS with dwell time of no more than 3 wk, over customized bariatric stents.

Cardiac septal defect occluder

The non-surgical closure of a cardiac septal defect was first described in 1976 and was considered a revolution in the treatment of cardiac defects[58]. Although developed for the closure of cardiac defects, the off-label use of the CSDO has been reported for the management of bronchopleural and GI fistulas [59].

The CSDO is a self-expanding, double-disc closure device composed of nitinol and polyester, with shape-memory and impressive expansion force (Figure 8)[28,59]. The thick waist portion serves to self-center the device during deployment to close the defect. The disc diameter varies from 9 mm to 54 mm, and the waist size varies from 4 mm to 38 mm.



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Figure 8 Cardiac septal defect occluder for the treatment of chronic fistulas after bariatric and metabolic surgery. A: Endoscopic imaging of a successful closure of a gastrocutaneous fistula after cardiac septal defect occluder (CSDO) placement in a distal staple line leak after laparoscopic sleeve gastrectomy; B: Methylene blue test showing no leakage to the skin; C: CSDO migration after placement in an early leak after Roux-en-Y gastric bypass surgery. CSDO: Cardiac septal defect occluder.

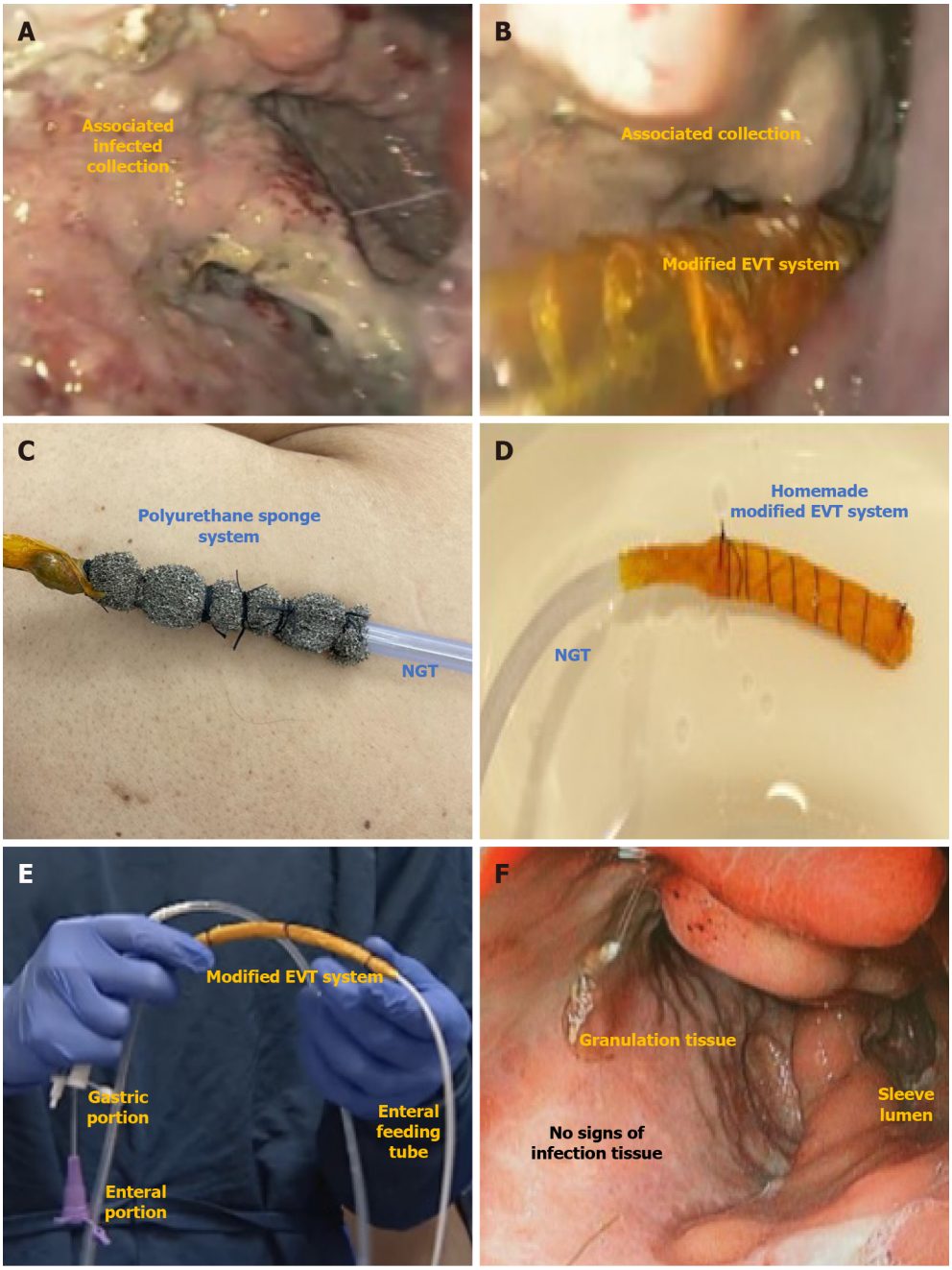
The appropriate device size ("waist diameter") should be 50% larger than the defect orifice. Regarding placement, first, the distal flange is released either into the GI lumen (percutaneous placement) or the fistula tract (endoscopic placement), then, after adequate position is confirmed, the proximal flange is deployed[28].

In a multicenter study, clinical success rate for late and chronic defects after BMS was 97.1% *vs* 62.5% in acute and early scenarios[28]. Due to its expansion force, immediate contrast study or methylene blue test after the procedure usually confirms complete occlusion of the fistulous tract allowing hospital discharge after anesthesia recovery (Figure 8A and B)[32]. If immediate, complete closure fails, adjunctive therapies such as glues/tissue sealants can be performed[31]. Therefore, CSDO is recommended for late and chronic defects as an epithelialized tract is needed for device accommodation. The device should not be used in acute and early leaks or fistulas as it can increases the size of the defect due to its significant expansion force (Figure 8C)[3].

In our experience, the CSDO is the best approach for epithelialized fistulas (Figure 5B) tracts but as an off-label high-cost device, we only use it after conventional technique failure. Regarding long-term follow-up, some devices stay in place and other migrate after complete healing of the defect[28,60].

Endoscopic vacuum therapy

EVT is indicated for GI transmural defects (Figure 9). Its high efficacy is associated with its mechanism of action that involves micro-deformation, macro-deformation, changes in perfusion (angiogenesis), exudate control, and bacterial clearance[4]. The traditional technique involves the use of a polyurethane sponge connected to a nasogastric tube placed into the extraluminal compartment (intracavitary position) or into the GI lumen (intraluminal position). The nasogastric tube is then connected to a vacuum machine with continuous negative pressure (between -125 and -175 mmHg). It is important to understand that, when an associated collection is identified (Figure 9A), the EVT system must be placed inside the collection (intracavitary) (Figure 9B). Despite the high efficacy, the traditional system (Figure 9C) is associated with challenging placement and removal (through the mouth) leading to a prolonged procedure, and the need for frequent exchanges due to tissue ingrowth.

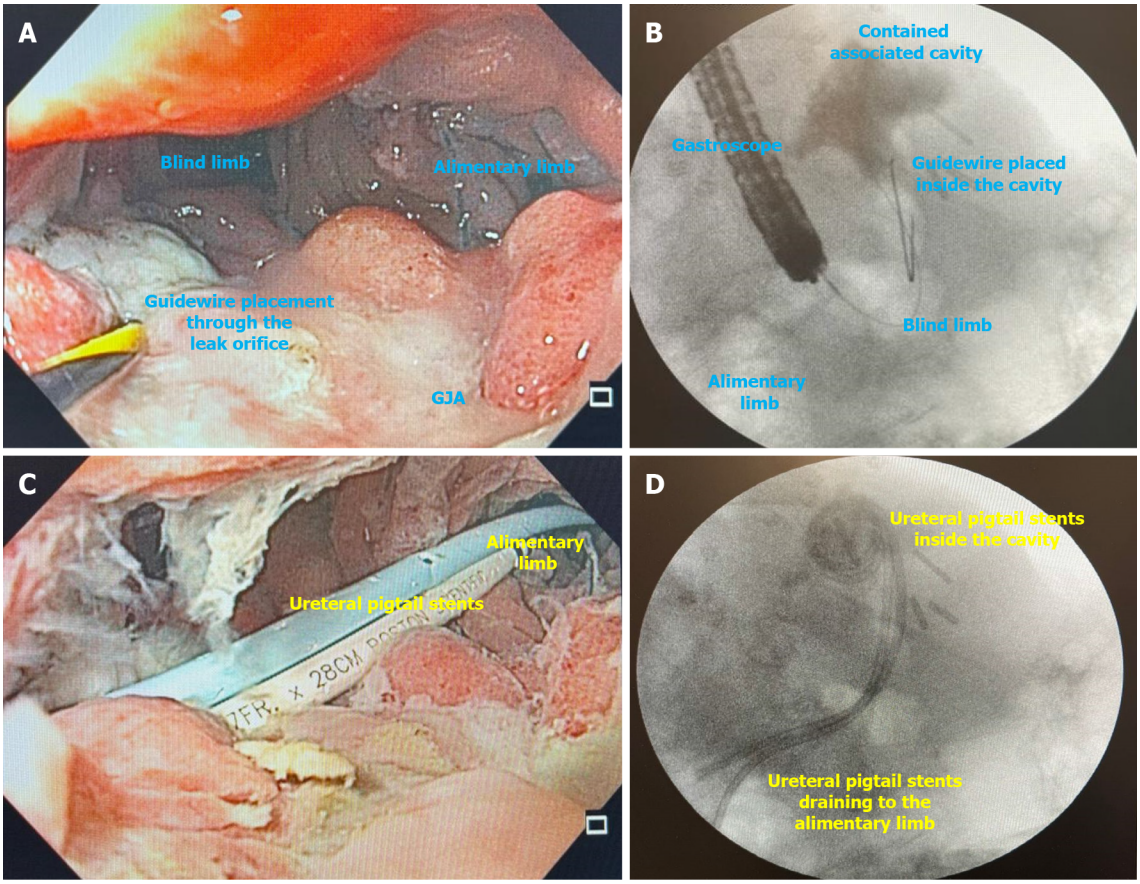


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Figure 9 Endoscopic vacuum therapy for the treatment of a post bariatric and metabolic surgery leak. A: Leak associated collection after laparoscopic sleeve gastrectomy; B: Intracavitary modified endoscopic vacuum therapy (EVT) placement; C: Traditional (polyurethane) sponge system; D: Homemade modified EVT; E: Triple lumen tube used for intraluminal EVT allowing for drainage and nutrition with one tube through the nares; F: Complete tissue healing showing granulation tissue and no signs of infection after EVT treatment. EVT: Endoscopic vacuum therapy; NGT: Nasogastric tube.

To overcome these limitations, the open pore film system was developed showing several benefits compared to the traditional sponge system, maintaining a high efficacy and safety profile. These advantages include easy placement (through the nares), reduction in procedure time, longer interval between EVT system exchanges, and a lower rate of AEs[61]. Despite these improvements, the high cost and limited availability of the open pore film remain barriers to its use and widespread adoption. Recently, a cost-effective modified-EVT manufactured from widely available materials and utilizing wall suction was described with promising results (Figure 9D)[62-65]. Furthermore, the use of intraluminal EVT *via* a triple-lumen tube was reported that allowed both drainage (gastric fenestrations) and nutrition (enteral portion) using a single tube through the nares (Figure 9E)[64,66].

Previous studies had already demonstrated very positive outcomes for EVT in the management of general upper GI leaks and fistulas, including with higher success rates and lower AEs rates when compared to SEMS[67]. A recent meta-analysis evaluated the use of the EVT in the management of leaks and fistulas after BMS demonstrating a pooled clinical success rate of 87.2% with 6% AE rate. In this



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Figure 10 Endoscopic internal drainage with double pigtail stents. A: Leak orifice at the gastrojejunal anastomosis suture line; after Roux-en-Y gastric bypass surgery; B: Fluoroscopy showing a contained associated collection and guidewire placement; C: Endoscopic internal drainage with ureteral pigtail stents; D: Fluoroscopy showing adequate placement and drainage of the leak associated collection. GJA: Gastrojejunal anastomosis.

study, the mean number of EVT system exchanges was 6.47 with a mean interval of 4.39 d[68]. Despite the satisfactory results, there is a concern regarding patients' complaints due to the nasogastric tube and a possible prolonged hospital stay. Additionally, the risk of major bleeding related to the development of a fistula between the wound cavity and major blood vessels, including the aorta or its branches is a reported concern from some experts[4,69,70].

In our practice, the cost-effective modified EVT is the preferred approach due to its high efficacy, satisfactory patient acceptance, and safety benefit of avoiding sponge dislodgement and exchange related bleeding[71]. Based on our experience, the best indication for EVT is acute and early leaks with associated infected collections. In these cases, after clinical improvement and collection clearance associated with granulation tissue (Figure 9F), we do change the intracavitary EVT for EID with DPS allowing earlier hospital discharge or when the associated cavity is smaller than 3 cm, we change the intracavitary EVT for intraluminal EVT.

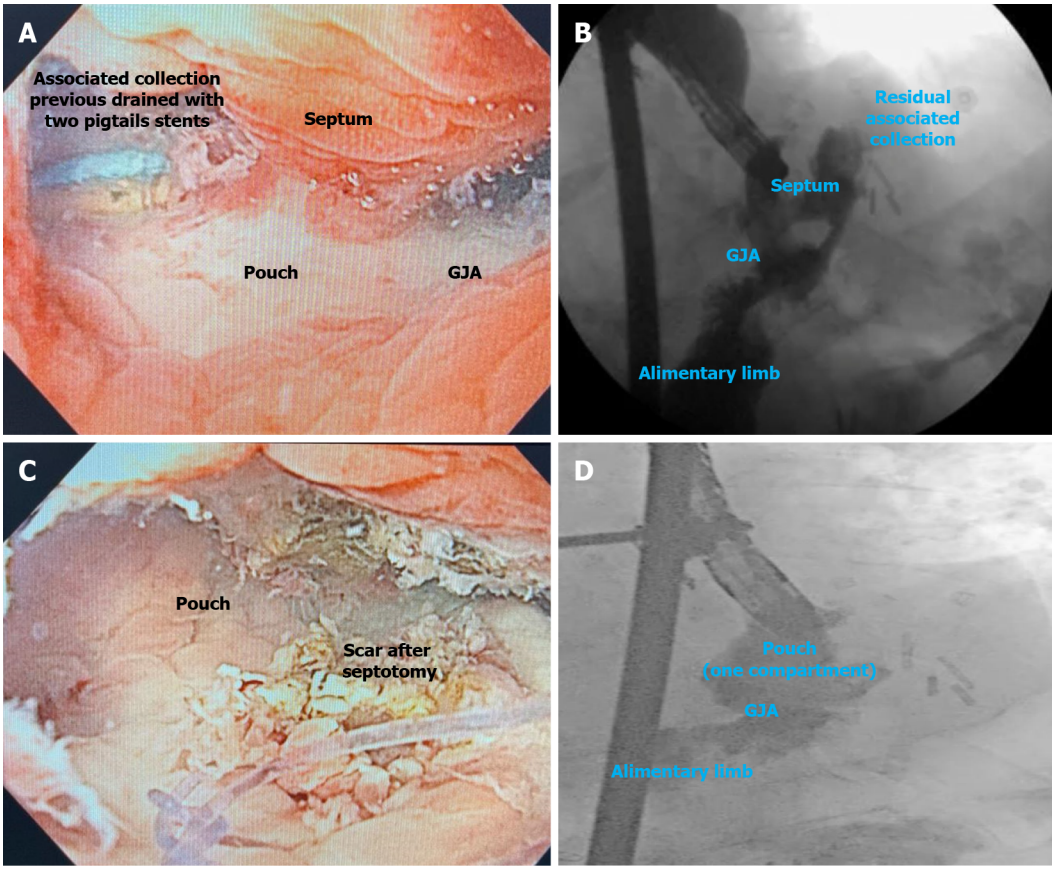
Endoscopic internal drainage with double pigtail stent

EID with DPS (Figure 10) is indicated for leaks and fistulas with associated fluid collections after BMS. The principle for internal drainage is based on the concept that when the pressure within the gastric lumen is lower than that of the perigastric collection, flow will be directed into the GI tract. Additionally, oral contents will preferentially flow through the gastric lumen.

For EID with DPS to be effective as a sole strategy, the following conditions should be absent: (1) Uncontained perigastric collections; (2) High intragastric pressure secondary to downstream stenosis; and (3) Existence of a gastropleural fistula. With drainage, the perigastric cavity, will typically contract until it is obliterated, resolving the leak. When evaluating the response to therapy, the ability to tolerate diet as well as a decrease in the size of the perigastric cavity are indicative of clinical success[72,73].

EID has been widely adopted, especially in Europe, with high clinical success rates for both acute and chronic leaks and fistulas and a low rate of AEs, allowing a shorter hospital stay.

The largest study evaluating EID with DPS for the treatment of complications after BMS included 617 patients, with a clinical success rate of 89.5% for leaks and 78.5%, for fistulas[74].



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Figure 11 Septotomy. A: Septum between the associated collection and the leak orifice at the gastrojejunal anastomosis staple line after Roux-en-y gastric bypass surgery; B: Fluoroscopy showing a septum between the leak associated collection and the gastric pouch; C: Endoscopic image after septotomy; D: Fluoroscopy showing a gastric pouch without associated collections as the septotomy transformed the pouch and the associated collection in one compartment. GJA: Gastrojejunal anastomosis.

Placement of DPS is easy to learn (Figure 10A and B), demonstrated by high clinical success when performed by an endoscopist with (84.71%) or without (83.41%) experience in EID as demonstrated by a recent systematic review[72]. Additionally, it also presents satisfactory clinical success as a rescue therapy (78.05%)[72].

Defect closure is usually achieved after a long period of treatment. However, as most patients are not hospitalized, present no symptoms, receive oral diet, and return to their daily activities, the long treatment time is not considered an issue when compared to other techniques which are associated with symptoms related to the therapy, such as bariatric stents, or need for hospital stay, such as EVT[72].

AEs are reported in about 13.8% of patients, mostly due to stent migration, but also including perforation and bleeding[72].

In our experience, EID with DPS is the best approach for defects with associated collections in patients with no signs of sepsis. As most patients are not hospitalized, close follow-up is needed to avoid complications. Additionally, to minimize the risk of AEs related to the DPS, we prefer to use ureteral stents as they are more flexible and softer than biliary DPS, avoiding damage to tissue and vessels (Figure 10C and D)[75].

Septotomy

Septotomy (Figure 11) must be performed when a septum between the defect orifice/associated collection and the GI lumen is identified (Figure 11A and B). The principle of this therapy is similar to the Zenker´s diverticulotomy. The septum is sectioned to match the pressure of the leak or fistula orifice within the gastric chamber, providing better drainage (Figure 11C and D). The septotomy can be performed with endoscopic electrosurgical knives, through the scope scissors or argon plasma coagulation (APC), until the depth of the suture line without exceeding this limit (the cut must not extend beyond the base of the perigastric cavity) to avoid perforation[3,76]. It is important to state that in most cases, several sessions are needed.

Septotomy is associated with high clinical success rates and with low AEs in expert hands. In a study involving 27 patients with leaks and fistulas after BMS, including RYGB, LSG, and duodenal switch, the clinical success rate after one to six septotomies was 100%, with an average treatment time of 18.11 d

Table 4 Suggested recommendations on management of post bariatric surgical leaks and fistulas

Leak or fistula characteristics + patient clinical condition	Recommended management (first line approach)	Possible therapy (second line approach)	Possible endoscopic therapies based on defect characteristics
Acute and early leaks with undrained uncontained collection in unstable patients	Surgical lavage + external drainage (surgical placement) ± surgical repair ± endoscopic therapy (see column 4)	Image-guided external drainage + endoscopic therapy (see column 4) OR Intracavitary EVT	Defect < 2 cm: Cap mounted clips OR stents OR intraluminal EVT; Defect > 2 cm: Stents OR intraluminal EVT; If a septum is diagnosed (early): Septotomy must be performed
Acute and early leaks with undrained uncontained collections in stable patients (rare condition as most patients with undrained uncontained collections presents with peritonitis/sepsis)	Image-guided external drainage + endoscopic therapy (see column 4)	Surgical lavage + external drainage (surgical placement) ± surgical repair ± endoscopic therapy (see column 4) OR Intracavitary EVT	Defect < 2 cm: Cap mounted clip OR stents (prefer stents if associated with downstream stenosis) OR intraluminal EVT; Defect > 2 cm: Stents OR intraluminal EVT; If a septum is diagnosed (early): Septotomy must be performed
Acute and early leaks with undrained contained collections (both unstable or stable patients - most of these patients are stable due to the contained collection)	Endoscopic drainage techniques: Intracavitary EVT OR EID with DPS; If a septum is identified, septotomy must be performed	Image-guided external drainage + endoscopic therapy (see column 4)	Defect < 2 cm: Cap mounted clips OR stents (prefer stents if associate with downstream stenosis) OR intraluminal EVT; Defect > 2 cm: Stents OR intraluminal EVT; If a septum is diagnosed (early): Septotomy must be performed
Late and chronic leaks (both unstable or stable patients - most of these patients are stable as uncontained collection are extremely rare in late and chronic leaks)	Endoscopic drainage techniques: EID with DPS OREVT (intracavitary if associated collection > 3 cm); If a septum is identified, septotomy must be performed	Image-guided external drainage + endoscopic therapy (see column 4) OR Surgical approach	Defect < 2 cm: Cap mounted clips OR CSDO OR tissue sealants/glues (as an adjunctive therapy); Defect > 2 cm: CSDO OR tissue sealants/glues (as an adjunctive therapy)
Late and chronic fistulas (both unstable or stable patients - most of these patients are stable)	Endoscopic therapy (see column 4); Cytology brushing or APC to loosen granulation tissue before endoscopic therapy is helpful; If a septum is identified, septotomy must be performed	Surgical approach	Defect < 2 cm: CSDO ± tissue sealants/glues OR tissue sealants/glues ± cap mounted clips OR tissue sealants/glues + intraluminal EVT; -Defect > 2 cm: CSDO ± tissue sealants/glues OR tissue sealants/glues + intraluminal EVT,
Late and chronic gastro-gastric fistula	Defect < 10 mm: Endoscopic therapy (see column 4); Defect > 10 mm: Surgical approach	Surgical approach after endoscopic management failure	APC ± CSDO ORAPC + suturing OR APC + cap mounted clip

APC: argon plasma coagulation; CSDO: Cardiac septal defect occluder; DPS: Double pigtail stents; EID: Endoscopic internal drainage; EVT: Endoscopic vacuum therapy.

[77]. Our experience reflects the results of this study. We do prefer to use APC as it is associated with a low rate of bleeding.

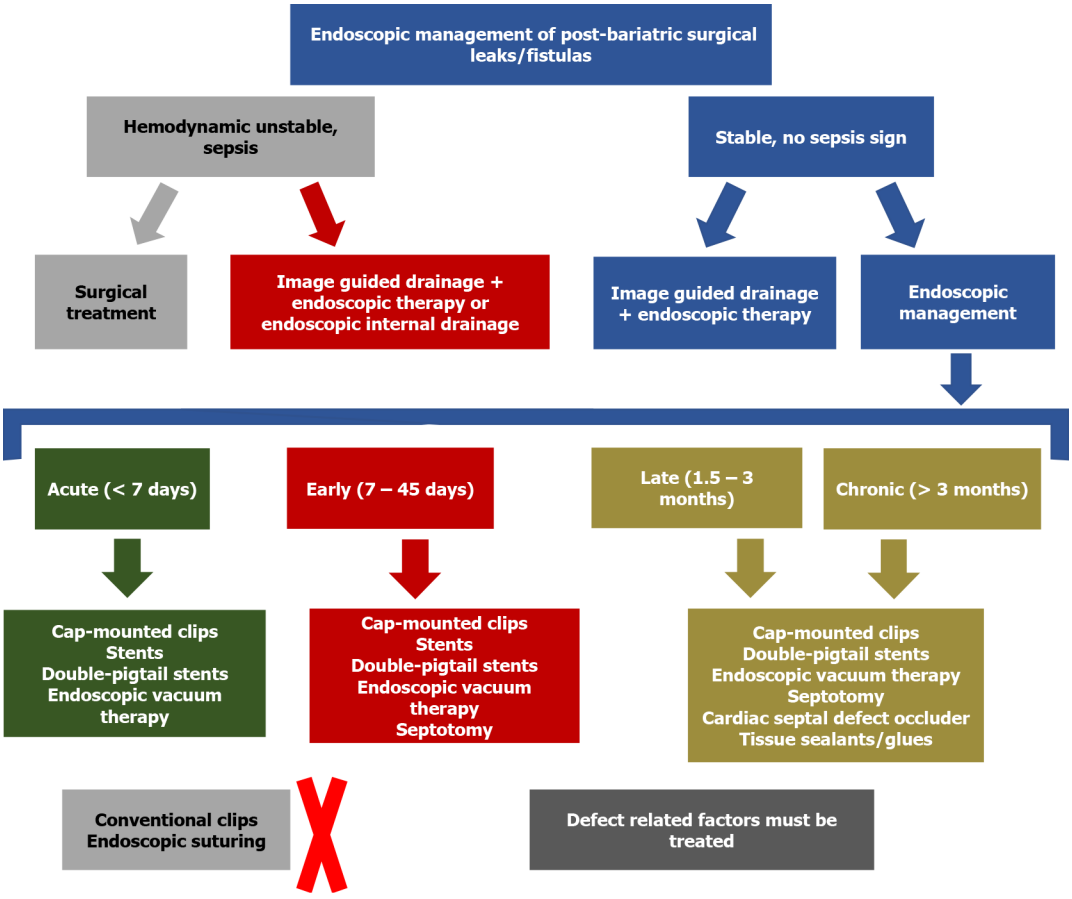
In our practice, the presence of a septum is the most common cause of late and chronic leaks and fistulas refractory to endoscopic treatment in non-specialized centers referred to our institution. Therefore, when treating a leak or fistula after BMS, attention to the presence of a septum and need for septotomy is required to achieve clinical success.

CHOOSING THE BEST APPROACH

Based on the pathophysiology of leaks and fistulas after BMS, the appropriate therapy should be individualized, considering the patient´s clinical condition, defect characteristics (size, chronicity, associated collections, and health of the surrounding tissue), device availability, cost, patient preference, mechanism of action of each endoscopic therapy, one's personal experience and comfort and local availability of experienced physicians. Although there is a paucity of high-quality studies to provide standardized treatment algorithms, we summarize our recommendations on the management of these challenging conditions in Table 4 and Figure 12 based on our large referral volume, and review of the available literature.

DISCUSSION

The management of post bariatric surgery complications is challenging as patients have obesity-mediated chronic pro-inflammatory state and are in a catabolic state related to weight loss. Additionally, most patients have comorbidities, such as T2DM, arterial hypertension, among others. The most feared complication is leak, as an infection in these patients can be life-threatening or severely



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Figure 12 Suggested algorithm for the management of post bariatric surgical leaks and fistulas.

morbid, especially when early identification and management is not achieved. Although laboratory tests and imaging are important for diagnosis, patient clinical conditions must be the primary consideration. If a patient presents with tachycardia, fever, and intense abdominal pain, a surgical revision should not be postponed.

In cases of acute and early leaks associated with infection, there can be a need for admission to an intensive care unit and long-term hospital stay is often needed. Additionally, some patients cannot take an oral diet and there is a need for enteral nutrition or parenteral nutrition. Unfortunately, long-term inpatient related diseases such as pneumonia, deep venous thrombosis, healthcare-associated infections, and others, can affect these patients. Therefore, multidisciplinary care is required, including surgeons, endoscopists, radiologists, intensivists, infectious disease experts, nutrition experts, nurses, physiotherapists, and other specialists contributing to successful outcomes. Ideally, these patients should be treated in a tertiary referral center given the complexity and relative rarity of this disease state.

For late and chronic leaks and fistulas, almost all patients present in a stable condition, most of them need several procedures and longer treatment time to achieve defect closure. Despite the need for more sessions, as these patients are stable, they do not need to be hospitalized in most cases and can be discharged to attend subsequent elective endoscopic procedures while they carry on with their normal activities.

Endoscopy has evolved to become first line therapy for the treatment of post-bariatric leaks and fistulas, except in unstable patients with undrained, uncontained collections (peritonitis) where a surgical approach is preferred. There are several available endoscopic devices and techniques with different mechanisms of actions, including closing, covering, and draining therapies. When determining the appropriate endoscopic technique, fundamental principles must be considered, such as draining of associated collections and treatment of related factors such as dilation of downstream stenosis and foreign body removal. It is mandatory that endoscopists incorporating management of BMS complications into their clinical practice have expertise in several advanced therapeutic endoscopic techniques as well as knowledge in using fluoroscopy, managing percutaneous drains, and also understanding of post-surgical anatomy and pathophysiology concepts for origin and maintenance of leaks and fistulas. It is also important to understand that there is no gold standard approach for these patients and usually more than one endoscopic intervention is required, especially in a chronic scenario. Furthermore, combined therapies may be required for some patients to achieve clinical success.

CONCLUSION

In summary, a multidisciplinary team and individualized evaluation considering patient and defect characteristics, available resources, local and personal experience, and knowledge of fundamental principles and mechanisms of action of each technique is essential to choosing the best approach for the management of post-bariatric surgical leaks and fistulas. With a committed approach, high rates of leak and fistula closure can be achieved.

FOOTNOTES

Author contributions: de Oliveira VL, Bestetti AM, Trasolini RP, de Moura EGH, de Moura DTH performed the conception and design of the work; de Oliveira VL, de Moura DTH, Bestetti AM drafted the manuscript; de Oliveira VL, Bestetti AM, Trasolini RP, de Moura EGH, de Moura DTH contributed to the critical review of the manuscript for important intellectual contents; de Moura DTH, Trasolini RP, de Moura EGH contributed to the manuscript supervision; Trasolini RP revised the manuscript for English language polishing requirements; de Oliveira VL, Bestetti AM, Trasolini RP, de Moura EGH, de Moura DTH contributed to the approval of the version to be published, have participated in conceptualizing the research or content of the manuscript, in writing or critically editing the manuscript, and/or in analysis of data presented in the manuscript; Consent to submit has been received from all co-authors.

Conflict-of-interest statement: Dr. Eduardo Guimarães Hourneaux de Moura reports personal fees from Boston Scientific, personal fees from Olympus, outside the submitted work; Dr. Diogo Turiani Hourneaux de Moura reports personal fees from Bariatek, outside the submitted work; The other authors reported no potential conflict of interest.

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Country/Territory of origin: Brazil

ORCID number: Victor Lira De Oliveira 0000-0002-5132-0309; Alexandre Moraes Bestetti 0000-0002-5358-2169; Roberto Paolo Trasolini 0000-0001-8059-9807; Eduardo Guimarães Hourneaux de Moura 0000-0003-1215-5731; Diogo Turiani Hourneaux de Moura 0000-0002-7446-0355.

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MINIREVIEWS

Advances in acute and chronic pancreatitis

Williamson B Strum, Clement Richard Boland

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Williamson B Strum, Department of Gastroenterology, Scripps Clinic, La Jolla, CA 92037, United States

Clement Richard Boland, Department of Medicine, University of California San Diego, La Jolla, CA 92037, United States

Corresponding author: Williamson B Strum, MD, Research Scientist, Department of Gastroenterology, Scripps Clinic, No. 10666 N Torrey Pines Road, La Jolla, CA 92037, United States. williamsonstrum@yahoo.com

Abstract

Acute pancreatitis (AP) and chronic pancreatitis are the third leading gastrointestinal causes for admissions and readmissions to hospitals in the United States. This review of articles published between 2019-2022 (December) from international sources identified four categories of crucial new findings: The report includes (1) New genetic pathogenic mutations (*TRPV6*); expected genetic outcomes in a Northern European population; (2) a new serum diagnostic marker for AP-fatty acid ethyl esters-distinguishing acute pancreatitis associated with alcohol; explanations of the impact of monocytes/macrophages on the inflammatory process that defines their future in diagnosis, staging, and treatment; (3) innovations in timing of per os low-fat, solid food intake immediately on admission; resolution of concepts of aggressive parenteral fluid intake; dramatic shifts to nonoperative from operative treatment of infected pancreatic necrosis. Each modification reduced interventions, complications, and lengths-of-stay; and (4) authoritarian recommendations for medical treatment of chronic pain. These advances offer opportunities to initiate newly proven treatments to enhance outcomes, alter the natural history, and envision the future of two diseases that have no known cure.

Key Words: Genetics; New diagnostics; Macrophage regulation; New approaches to early feeding; Fluid management

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Core Tip: Acute and chronic pancreatitis are leading causes for admissions to hospitals. This review identified four categories of crucial new findings including: (1) New genetic mutations (*TRPV6*); expected genetic outcomes; (2) new serum diagnostic markers-distinguishing pancreatitis associated with alcohol, and defining the impact of monocytes/macrophages on the inflammatory process; (3) critical innovations: In timing of PO low-fat, solid food intake immediately on admission; resolved concepts on fluid intake; non-operative treatment of infected necrosis; and (4) authoritarian recommendations for treatment of chronic pain. These advances offer opportunities to initiate newly-proven treatments to enhance outcomes and alter the natural history.

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INTRODUCTION

Acute pancreatitis (AP) and chronic pancreatitis (CP) are two of the most common causes for gastrointestinal-related office visits and admissions to hospital[1-3]. AP and CP are multifactorial diseases in which the former may progress to the latter as chronic inflammation and autodigestion lead to fibrosis and atrophy. Information that improves understanding of the diseases is critical to advancing therapy. The objective of this review was to identify pivotal studies that directly impart usable information relative to pathophysiology, diagnosis, preventative and other treatments.

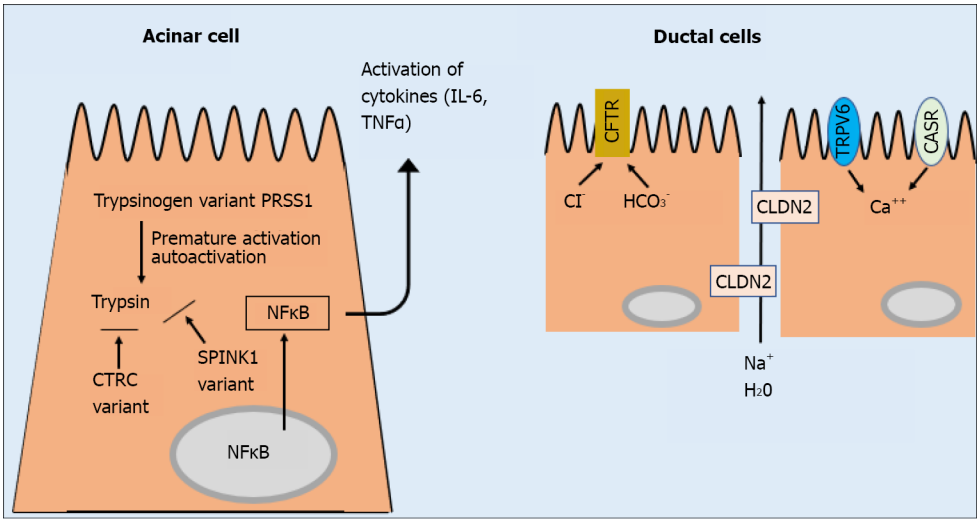
METHODS

The information in this review was obtained between 2019 through December 2022 from PubMed, Ovid, International Guidelines, expert opinion, and personal contact with experts. We looked specifically for well-designed, randomized, controlled original research, systematic reviews, meta-analyses, expert guidelines, and professional association guidelines addressing changes favorably affecting outcomes. Case reports, case series and others considered irrelevant to the study were excluded as were most articles outside the inclusive dates, although some were included for supporting information. Over 400 articles were reviewed for possible relevance, with primary selections based on new information considered important and usable for health care workers.

OBSERVATIONS AND DISCUSSION

Pathophysiology: Genetic risks and clinical characterization

The landmark discovery of mutations in the pancreatic digestive genes coding for trypsinogen (*PRSS1* and *PRSS2*[4] catalyzed a search for additional pathologic variants in pancreatic digestive genes. The search has been rewarded with the discovery of a sufficient number of genetic variants to permit a mechanistic classification suggesting their pathogenic effect[5-7]. These include genetic variants in: (1) The trypsin-dependent pathway, including *PRSS1*, *PRSS2*, *SPINK1*, and chymotrypsin C (*CTRC*) genes; (2) the ductal physiology pathway including the cystic fibrosis transmembrane conductance receptor (*CFTR*), *claudin* 2 (*CLDN2*), possibly the *calcium sensing receptor* (*CASR*); proposed, and offered on many testing panels, but still controversial), plus newly discovered variants in the calcium channel coding genes (*TRPV6*, not yet on most testing panels)[8]; and (3) the misfolding-dependent pathway including a distinct subset of *PRSS1* variants, carboxypeptidase A1 (*CPA1*), and rare variants of carboxyl ester lipase (*CEL*) (Figure 1). The *TRPV6* variants are important new additions to the ductal-pathway genes, which alter calcium concentration in ductal fluid. The *TRPV6* variant is identified in 4.3% of nonalcoholic CP patients in Japan and 2.0% in Europe, indicating a substantial global risk gene for CP. Most of the carriers were heterozygous for the variant, implicating the potent pathogenic effect of certain *TRVP6* variants (*i.e.*, haploinsufficiency). Furthermore, 20% with defective *TRPV6* variants were transheterozygous for *SPINK1* variants, an unusually high association[8,9]. More than half of the *TRPV6* variants cause subtle functional defects with loss-of-function for removing calcium from the ductal fluid, leading to increased ductal calcium concentrations. This discovery offers a possible novel target for therapeutic intervention since *TRPV6* expression is regulated by 1-alpha,25-dihydroxyvitamin D3, and partial correction of *TRPV6* dysfunction in heterozygous carriers might be feasible with vitamin D supplements[9]. Collateral laboratory studies demonstrate that much of the injury in acute and chronic



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Figure 1 Diagrammatic depiction of fundamental events contributing to the complex molecular pathways resulting in pancreatitis. Although a unifying concept is not available for all events, recent literature has provided a clearer description of the complexity of pathologic events which is briefly summarized. A proposed first step is injury to cytoplasmic organelles from external stressors, including prolonged, high intake of ethanol and smoking. These injuries result in endoplasmic reticulum stress, mitochondrial depolarization, inadequate ATP production, vacuole accumulation, protein misfolding, and disordered autophagy leading to activation of trypsinogen and inflammatory pathways. Disruption of the normal pathways may be augmented by genetic mutations at key functional sites responsible for trypsinogen activation, trypsin inhibition, Ca^{++} concentrations, and $HCO3^-$ and H_2O movement[4-9,20,21]. CTRC: Chymotrypsin C; CFTR: Cystic fibrosis transmembrane conductance receptor; NF-Kb: Noncanonical nuclear factor-kappaB; TNF-α: Tumour necrosis factor alpha; IL: Interleukin.

pancreatitis evolves from alterations of acinar and ductal digestive function by these pancreatitis-susceptibility mutants, culminating in disruption of the protease-antiprotease interaction in the serine protease trypsinogen pathway within pancreatic acinar and ductal cells[5-9].

Recent studies by Lewis *et al*[10] provide clinical characterization of genetic risks for CP in a North American population of European ancestry. Patients with early-onset (< 35 years) idiopathic CP and no alcohol exposure had significantly more pathogenic variants in 49% of cases compared to no more than 26% in those with late-onset idiopathic CP or CP associated with light-to-moderate or heavy-to-very heavy drinking. Likewise, the early-onset group was significantly more likely to have pathogenic variants in *SPINK1* (24%) or *CFTR* (36%) than patients with late-onset idiopathic CP (9% and 15%) or CP associated with light-to-moderate drinking (7% and 19%) or heavy-to-very heavy drinking (9% and 13%). Evidence supporting pathogenicity of the mutants included a younger age of onset of symptoms in which the *SPINK1* mutation decreased the age at onset to 12 years from 24 years in early-onset idiopathic CP ($P = 0.004$) and to 24 years from 50 years in the light-to-moderate drinkers ($P < 0.001$). The *CFTR* mutation dropped the age at onset to 41 years from 50 years in the light-to-moderate drinkers ($P = 0.030$). Sixty-one cases of early-onset ICP were characterized by persons with an average age of 20 years, incessant pain, pancreatic calcifications, and genetic variants in the *SPINK1*, *CTRC*, or *CFTR* genes in almost half the cases. These collective findings reclassified as many as 23%-49% of cases of ICP to those with potential genetic causes.

CFTR was the most common gene with pathogenic variants in 13%-36%. It is important to recognize that interactions between incompletely penetrant genetic variants-including several working synergistically (*i.e.*, epistasis) and a pronounced exposure to alcohol, smoking, or other toxins are frequently involved in the final pathways leading to acute and chronic pancreatitis. More often than not, multiple factors are responsible. The pathologic progression then gradually leads to well-recognized complications of chronic inflammation with fibrosis, ductular calcifications, pain, steatorrhea, insulin insufficiency, fluid collections, disability, and premature death.

Along with appropriate counseling, testing for the six (or seven including the newly described *TRPV6* variants) commercially evaluable genetic causes of acute and chronic pancreatitis is recommended in cases that have age of onset < 35 years, relapsing pancreatitis, or are idiopathic. The results offer valuable information regarding diagnostic probability of a genetic predisposition, a prediction as to probable cofactors, how to initiate available treatment as early in the process as possible, and do not require repetitive testing. Persons who have extensive comorbidities or are elderly may not benefit from genetic investigation.

Acute pancreatitis: New tools for diagnosis

AP, a disease with a variety of etiologies and risk factors is diagnosed by blood levels of amylase and lipase, abdominal pain, and imaging abnormalities of the pancreas[11-13]. A new diagnostic test has produced intriguing results. Vela and colleagues conducted a prospective, blinded trial comparing

blood levels of fatty acid ethyl esters (FAEEs), nonesterified fatty acids, and ethanol in 175 patients at the time of hospitalization with alcoholic pancreatitis, alcoholic intoxication, nonalcoholic pancreatitis, and normal controls[14]. Distinguishing differences were that median FAEEs were similarly elevated in alcoholic pancreatitis (103 nmol/L) and alcoholic intoxication (205 nmol/L), whereas there was no significant elevation in nonalcoholic causes of AP (8 nmol/L) or controls (1.7 nmol/L). By its unique ability to demonstrate diagnostic elevations of FAEEs in association with alcohol intake, this test could become an efficient tool for identifying alcohol as the cause in alcohol-associated pancreatitis when present and eliminating alcohol as the culprit when caused by other factors. That alcohol intoxication without AP raises the level of FAEEs is explained by a well-known effect of excessive ethanol ingestion on cell membranes. The study did not distinguish between AP and alcohol-induced AP superimposed on CP, although the latter would be suspected in most of the latter cases.

In a similar vein, Manohar *et al*[15] have presented data defining the complex role of immune responses in monocytes and macrophages using single-cell mass Cytometry by Time Of Flight in two experimental models and in patients with acute, recurrent, and severe AP. As inflammatory cells determine disease severity and pancreatic damage is increased by tissue-derived neutrophils, macrophages are the predominant immune cell in the early phases of AP. Their report explains the role of the immune response in AP, provides insight toward the biological significance of novel Ly6Gc+/CD206+ monocytes and macrophages, and shows how these cellular components may be used to diagnose AP, define its severity, and target potential treatment[15,16].

Chronic pancreatitis: Alcohol and smoking

CP is diagnosed by imaging scans showing glandular atrophy, ductal distortion, and calcifications, or by biopsy demonstrating widespread fibrosis, and is, like AP, characterized by a variety of similar etiologies and risk factors, excepting gallstones and medications[2,3,13]. The relationship of ethanol and smoking to CP is indisputable. Both the amount and duration of exposure matter, and the exposures may vary with time and gender. A contemporary analysis of daily ethanol consumption in persons with alcoholic CP revealed a median of 5.1 drinks/d, in which 12.0 g/drink = 61.5 g/d of ethanol[17]. Consumption of up to 110-277 g/d over 5-25 years may transpire by the time of diagnosis of alcoholic CP[2,3,17-19]. A lower intake of < 50 g/d is implicated where the disease progresses more slowly, and persons are older at the time of diagnosis with less pain and fewer complications[10]. Evidence substantiating the role of ethanol and tobacco intake in perpetuating the fibro-inflammatory phase is exemplified by unabated progression even if ethanol and tobacco intake are discontinued, although at a slower pace when compared with continued usage of both[2,3,10], implicating ethanol and tobacco activating subclinical pancreatic stellate cell fibrosis for years before becoming clinically apparent[19-21].

Recently, models ranging from necroptosis to pyroptosis show how a pathogenic metabolic pathway can provoke inflammation, often initiated by premature intrapancreatic activation of trypsinogen, and be perpetuated by synergy between genetic, epigenetic, immune and environmental factors[5-9] as clinical and post-mortem studies of chronic alcoholics without a sentinel AP event show pathologically recognizable CP in the form of fibrosis and/or ductal calcifications in 47%-68%, indicating extensive damage present before diagnosis[19,22,23].

NEW TREATMENTS

Early feedings in acute pancreatitis

Initiating early feeding per os or *via* nasogastric/nasojejunal routes beginning after the onset of AP is supported by multiple randomized controlled trials and has replaced prior practices of resting the gastrointestinal tract until pain subsides. This approach increases bowel motility and reduces rates of organ failure, infection, length of stay and mortality, substantiating the suspicion that an idle intestine leads to bacterial overgrowth, increased permeability and bacterial translocation[2,3,11,24]. Questions regarding preferred timing had remained but new results from a randomized controlled trial of 131 patients with mild-moderate AP provide clarity. The study compared those fed a low-fat, solid diet immediately upon decision for admission, regardless of symptoms or laboratory parameters, to those receiving conventional feeding with progressive diet as clinical and laboratory parameters improved. The results indicate that immediate feeding is safe and feasible with significantly decreased complications (4.2% *vs* 18.3%) and shorter length of stay (3.4 d *vs* 8.8 d) at one-half the costs[25].

Fluid resuscitation in acute pancreatitis

A major question addressing the appropriateness of early, aggressive hydration was just answered in an important multicenter, randomized, controlled trial from India, Italy, Mexico, and Spain that assessed the efficacy and safety of aggressive fluid resuscitation as compared with moderate resuscitation using lactated Ringer's solution in patients with AP; patients with moderate to severe disease or heart failure at baseline were excluded[26]. The primary outcome was the development of moderately severe or severe acute pancreatitis and secondary outcomes included organ failure and local complications

occurring after randomization and during the hospitalization. The aggressive-group received a bolus of 20 mL/kg body weight over 2 h, followed by an infusion rate of 3 mL/kg/h, and the moderate-group a dose of 1.5 mL/kg/h, with a bolus of 10 mL/kg/h if hypovolemic. Oral feeding was started at 12 h if the intensity of abdominal pain permitted. The first interim analysis included 249 of the planned sample size of 744 patients and uncovered significant fluid overload in the aggressive-resuscitation group at 20.5% *vs* 6.3% (P = 0.004), and the trial was stopped[26]. Significant differences were not reached for the primary or secondary endpoints, although the results tended to favor the moderate resuscitation group. An editorial analysis concluded that the trial results were stunning and irrefutable and that clinicians should follow an infusion rate of the moderate group with careful monitoring of volemic status, adding fluid if underhydrated or diuresis if overhydrated[27].

Pancreatic fluid collections

New data have created a decisive shift in treatment of infected necrotizing pancreatitis, in which antibiotics without drainage are replacing the long-held concept of antibiotics and immediate drainage of infection developing after AP[28]. The crucial substantiation came from a randomized, superiority trial in the Netherlands that compared immediate drainage of infected necrotizing pancreatitis *vs* postponement of drainage in patients with infected tissue occurring within 35 d from onset of initial symptoms of AP. Infection was documented by gram stain, culture, or clinical deterioration and both groups received antibiotics. The Dutch study enrolled 104 qualifying patients; complications and mortality were similar for both groups. However, the mean number of interventions was 4.4 for the immediately drained group compared to only 2.6 (40% less) for the deferred group; 37% (19/52) of patients in the deferred group did not require drainage, and the number of hospital days was shorter by 15% (51 d *vs* 59 d). The rationale for the study had been supported by retrospective studies that showed apparent safety in postponing intervention with drainage, although none have demonstrated superiority[28-30].

Current concepts for pain management in chronic pancreatitis

Pain affects most persons with CP and is the most significant obstacle to successful treatment.

Pain is the major cause of morbidity, disability, and impaired quality of life. Pain syndromes are complex, widely variable and range from severe and chronic to absent[31,32]. An up-to-date study of pain patterns in 1131 persons in 30 Dutch hospitals revealed continuous pain in 52%, intermittent pain in 20%, and no pain in 28%[33]. Patients with continuous pattern had more severe pain, used more opioids and neuropathic medications, and had lower quality of life.

Current concepts emphasize that pain may arise from origins other than the pancreas. Persistent, severe pain can transfer centrally in association with cerebral cortical thinning, central sensitization, central pain processing, or other neuropathology, and becomes less likely to respond to conventional measures. Pancreatic quantitative sensory testing that interrogates nociception and sensory response could help unmask irreversible central neuropathic changes that make interventional treatment of pancreatic pain less likely to be successful. During the course of disease, pain severity may increase or decrease as disease progresses over 10-25 years[31-33]. The initial treatment of choice for those who drink and/or smoke is complete abstinence to reduce the pain, prevent further episodes of AP, forestall progressive damage to the pancreas and lessen cancer risk to the pancreas and other organs[2,3,18,19,28-32]. Early diagnosis of the cause represents the first chance to initiate treatment to retard the progress of a disease that no one can cure. In cases of mild or intermittent pain, non-narcotic oral analgesics including NSAIDS, acetaminophen, tricyclic antidepressants, mirtazapine, and cognitive behavioral therapy may be helpful. Treatment with antioxidants may produce a small improvement, but pancreatic enzyme supplementation does not. Opioids are not recommended, due to their frequent and rising association with addiction and hyperalgesia, but may be considered after other reasonable options are exhausted[2,3,31-34].

In recognition that the longer pain is present, the more difficult it is to treat, a recent randomized surgical study of 88 patients (ESCAPE trial) compared early pancreatic ductal drainage by surgery for those with strong to weak pain of less than 2-6 mo *vs* conventional medical and endoscopic treatment. The initial results indicated a lower pain score in the surgical group of 37 *vs* 49 (P = 0.02)[35], similar to data from Cahen[35]. Additional endoscopic and surgical approaches have been addressed[36-41] but are beyond the scope of this review.

CONCLUSION

This narrative review captures the highlights of a number of significant articles published between January 2019 and December 2022 bearing new concepts in pathophysiology, diagnosis, and treatment of AP and CP. The major findings include clinical identification and application of new genetic information, newly identified serum biomarkers-FAEEs, for diagnosis of alcohol-related AP, novel concepts regarding monocyte/macrophage participation in the immune reaction, important acceleration in timing of early per os feedings in AP, answers to major questions concerning the appropriateness of

early, aggressive parenteral hydration, decisive shifts in non-operative management of infected pancreatic fluid, and updated, expert approaches to medical management of pain in CP. These advances are evaluated in the context of enhancing outcomes for these two acute and chronic inflammatory diseases.

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Country/Territory of origin: United States

ORCID number: Williamson B Strum 0000-0003-0213-2069; Clement Richard Boland 0000-0002-8120-5088.

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ORIGINAL ARTICLE

Case Control Study

Comparison of genomic and transcriptional microbiome analysis in gastric cancer patients and healthy individuals

Darja Nikitina, Konrad Lehr, Ramiro Vilchez-Vargas, Laimas Virginijus Jonaitis, Mindaugas Urba, Juozas Kupcinskas, Jurgita Skieceviciene, Alexander Link

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Darja Nikitina, Juozas Kupcinskas, Jurgita Skieceviciene, Institute for Digestive Research, Lithuanian University of Health Sciences, Kaunas 44307, Lithuania

Konrad Lehr, Ramiro Vilchez-Vargas, Alexander Link, Department of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University Hospital, Magdeburg 39120, Germany

Laimas Virginijus Jonaitis, Mindaugas Urba, Juozas Kupcinskas, Department of Gastroenterology, Lithuanian University of Health Sciences, Kaunas 44307, Lithuania

Corresponding author: Jurgita Skieceviciene, PhD, Professor, Senior Researcher, Institute for Digestive Research, Lithuanian University of Health Sciences, Eivenių g. 4, Kaunas 44307, Lithuania. jurgita.skieceviciene@lsmu.lt

Abstract

BACKGROUND

Helicobacter pylori and the stomach microbiome play a crucial role in gastric carcinogenesis, and detailed characterization of the microbiome is necessary for a better understanding of the pathophysiology of the disease. There are two common modalities for microbiome analysis: DNA (16S rRNA gene) and RNA (16S rRNA transcript) sequencing. The implications from the use of one or another sequencing approach on the characterization and comparability of the mucosal microbiome in gastric cancer (GC) are poorly studied.

AIM

To characterize the microbiota of GC using 16S rRNA gene and its transcript and determine difference in the bacterial composition.

METHODS

In this study, 316 DNA and RNA samples extracted from 105 individual stomach biopsies were included. The study cohort consisted of 29 healthy control individuals and 76 patients with GC. Gastric tissue biopsy samples were collected from damaged mucosa and healthy mucosa at least 5 cm from the tumor tissue. From the controls, healthy stomach mucosa biopsies were collected. From all biopsies RNA and DNA were extracted. RNA was reverse transcribed into cDNA. V1-V2 region of bacterial 16S rRNA gene from all samples were amplified and sequenced on an Illumina MiSeq platform. Bray-Curtis algorithm was used to

construct sample-similarity matrices abundances of taxonomic ranks in each sample type. For significant differences between groups permutational multivariate analysis of variance and Mann-Whitney test followed by false-discovery rate test were used.

RESULTS

Microbial analysis revealed that only a portion of phylotypes (18%-30%) overlapped between microbial profiles obtained from DNA and RNA samples. Detailed analysis revealed differences between GC and controls depending on the chosen modality, identifying 17 genera at the DNA level and 27 genera at the RNA level. Ten of those bacteria were found to be different from the control group at both levels. The key taxa showed congruent results in various tests used; however, differences in 7 bacteria taxa were found uniquely only at the DNA level, and 17 uniquely only at the RNA level. Furthermore, RNA sequencing was more sensitive for detecting differences in bacterial richness, as well as differences in the relative abundance of *Reyranella* and *Sediminibacterium* according to the type of GC. In each study group (control, tumor, and tumor adjacent) were found differences between DNA and RNA bacterial profiles.

CONCLUSION

Comprehensive microbial study provides evidence for the effect of choice of sequencing modality on the microbiota profile, as well as on the identified differences between case and control.

Key Words: Gastric cancer; Microbiome; *Helicobacter pylori*; 16S rRNA gene; 16S rRNA transcript; 16S rDNA

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Core Tip: In this study, we aimed to characterize the microbiota of gastric cancer (GC) on two levels: 16S rRNA gene and its transcript. Our study showed that only a small portion of bacterial sequences overlapped using those two approaches. Moreover, our study revealed that obtained results comparing the case group with the controls depend on the chosen modality. We also showed that *Reyranella* and *Sediminibacterium* was associated with the Lauren classification and RNA level was more sensitive to detect low abundant bacteria. This study provides novel insights into microbiome study as well as new founding related to complex GC pathogenesis.

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INTRODUCTION

Microbiota analyses are becoming increasingly relevant in scientific and clinical studies. Most modern microbiome studies use 16S rRNA gene sequence analysis at the DNA level, thereby enabling the identification of bacteria at all stages of their existence (active, dead, and inactive bacteria in the form of endospores) simultaneously. However, some of the more recent studies use RNA samples, which are subsequently reverse transcribed into cDNA for sequencing, giving us knowledge about the metabolic state of the microbial community[1]. RNA has a shorter half-life than DNA and turns over in cells more rapidly, providing a deeper look at bacterial activity[2].

The stomach has long been considered an almost sterile organ due to its acidic environment and enzymatic effects[3]. Since its identification, it is known that *Helicobacter pylori* (*H. pylori*) is perfectly adapted not only to survive in the acidic environment of the stomach, but also to colonize this part of the gastrointestinal tract[4]. *H. pylori* is the major cause of peptic ulcer disease and the most significant risk factor for gastric cancer (GC). GC remains one of the most common cancers in the world and the fourth leading cause of cancer-related death[5]. However, only a minority of people infected with *H. pylori* develop GC, which may be linked to non-*H. pylori* microbiota-associated alterations in the stomach[6]. Studies in insulin-gastrin (INS-GAS) mice and in humans indicated the importance of other members of the stomach bacterial community in the development of gastric carcinogenesis[7-10].

There is only one study that has compared DNA and RNA profiles of the stomach microbiota[11]. However, the profiles of the active and standing microbiota in GC have not been studied. In this study,

we systematically characterized the microbiota of GC on both levels using the 16S rRNA gene (DNA level) and its transcript (RNA level). GC tumor and tumor adjacent tissue samples, as well as healthy mucosa samples from the young control group, were used for the comparison. We obtained detailed data on bacterial composition within groups depending on study modality (DNA or RNA) and performed association analysis with clinical characteristics to question the potential impact of approach on the outcome.

MATERIALS AND METHODS

Study cohort

In total, 316 DNA and RNA samples from a group of 105 individuals were included in the study (Figure 1). The study cohort consisted of 29 healthy control individuals and 76 patients with GC. Participants did not report any antibiotic intake at least a month before endoscopy. Gastric tissue biopsy samples from damaged mucosa and healthy mucosa at least 5 cm from the tumor tissue were collected from GC patients using single-bite biopsy forceps. From the controls, healthy stomach mucosa biopsies were collected. Tissue samples were placed in sterile cryotubes (Thermo Fisher Scientific, United States), snap-frozen in liquid nitrogen, and stored at -86 °C until further study. Clinical data obtained from histological examination, such as tumor size, number of lymph nodes damaged by tumor cells, presence of metastases (TNM classification), cell differentiation (grading), type of GC (Lauren classification) and stage of GC, were included in the analysis. An overview of the demographic and clinical characteristics of the study cohort is given in Supplementary Table 1.

Study individuals were recruited at the Department of Gastroenterology at the Hospital of Lithuanian University of Health Sciences Kaunas Clinics during the years 2012-2018. This study was approved by the local ethics committee (BE-2-10), and all participants gave their written informed consent.

DNA, RNA extraction, cDNA synthesis and amplicon library preparation

Total DNA and RNA were extracted from gastric biopsy samples using an AllPrep DNA/RNA Mini kit (Qiagen, Germany) according to the manufacturer's recommendations. RNA was reverse transcribed into cDNA using the Superscript IV First-Strand Synthesis System Purification Kit (Invitrogen, Carlsbad, CA) and random hexamer primers, following the manufacturer's instructions. Amplicon libraries were generated as described previously[12,13]. The bacterial 16S rRNA gene V1-V2 region was amplified using the 27F and 338R polymerase chain reaction primers and sequenced on a MiSeq (2 × 250 bp; Illumina, Hayward, CA).

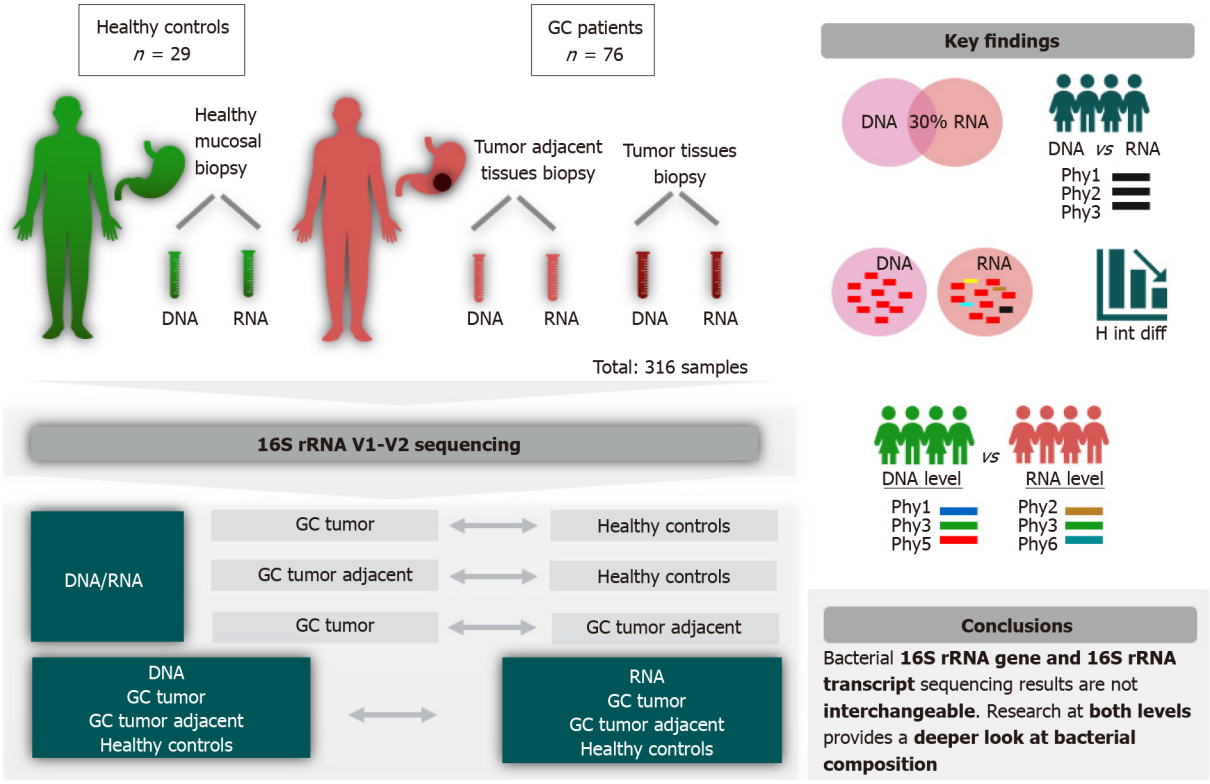
Bioinformatic and statistical analysis

Bioinformatic processing was performed as described previously[14]. FastQ files were analyzed using the dada 2 package[15], version 1.10.1, in R. In total, 7735281 paired-end reads were received, with an average of 22953 per sample. Samples that did not reach 5000 reads were discarded from the analysis (21 samples out of initial 337). All samples were rarefied to an equal sequencing depth of 5047 reads using the phyloseq package[16], with returning 10496 phylotypes (Supplementary Table 2). Phylotypes were annotated to a taxonomic affiliation based on the naive Bayesian classification[17] with a pseudo-bootstrap threshold of 80%. The relative abundances (expressed as percentages) of different microbial communities' phylogenetic ranks (from phylum to class, order, family, genus and phylotype) were used for downstream analyses.

The phylogenetic tree was built using the online tool iTOL[18], after hierarchical clustering using the Bray-Curtis algorithm[19] at the phylotype level in Past 3[20]. Bacterial richness and Shannon diversity indices were calculated using the vegan[21] package from R. The data matrices comprising the percentage of abundances of each of the abovementioned taxa were used to construct sample-similarity matrices by the Bray-Curtis algorithm, where samples were ordinated by principal coordinate analysis (PCoA) at the phylotype level using the patchwork[22] package from R.

Differences in relative abundance of detected bacteria (at all taxonomic ranks) between study groups were evaluated by PERMANOVA and ANOSIM statistical tests, using 9999 permutations. Groups were considered significantly different if the P value was < 0.05, considering an estimate effect-size F values for PERMANOVA and R values for ANOSIM tests. Calculation was made by Past 3 program. The distributions of taxa abundance values were compared by Mann-Whitney test followed by Benjamini-Hochberg correction for multiple comparisons, named as false discovery rate value. Differences were considered significant when the corrected p value (q value) was < 0.05.

The bacterial networks were visualized using Cytoscape 3.8.0[23], after the Spearman correlation test performed with the psych[24] package from R, with threshold of 0.2 in absolute value and P value < 0.05. Phylotypes that accounted for at least 1% of the total number of phylotypes and at least 10% of the samples in each group were used for correlation analysis.



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Figure 1 Study design illustration. GC: Gastric cancer.

RESULTS

General cohort

The bacterial contents of 180 biopsy samples taken from 105 individuals were characterized as described above (Supplementary Table 1). After sequencing and rarefying library size to the minimum sequencing depth, 10496 different phylotypes belonging to 23 phyla, 40 classes, 82 orders, 169 families, and 463 genera were retrieved and taxonomically annotated.

The global bacterial profiles were grouped into two clusters based on their Bray-Curtis similarities as percentages (Figure 2A). Analyzing all samples together, the main factor for clustering was bacterial heterogeneity. The first cluster consisted of samples with a more heterogeneous microbiome profile, where the most abundant bacteria accounted for less than 30%. All control samples (except T10_2) were located in this cluster. The second cluster - where the most abundant bacteria accounted for more than 30% of GC patient samples - was shaped by the most abundant bacteria *Helicobacter*, the abundance of which reached 98%-100% in some samples (Figure 2A, Supplementary Table 2).

Distinct profile of the gastric tissue microbiome at the RNA and DNA levels

Further PERMANOVA and ANOSIM analyses showed that DNA and RNA groups of the same study individuals were significantly different in all taxonomic ranks (Figures 3A-C; Supplementary Table 3). Differences between DNA and RNA samples were noticeable even at the phylum level. Although *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Fusobacteria* were the main bacterial phyla in all groups, *Bacteroides* and *Fusobacteria* were significantly more abundant at the DNA level. However, in the control group, *Firmicutes* and *Proteobacteria* were more abundant at the RNA level. Bacterial profile analysis indicated that only a portion of phylotypes (18%-30%) were common between bacterial profiles obtained from DNA and RNA samples (Figure 3D). PCoA supported the distinction of bacterial communities at the DNA and RNA levels, especially in the control group (Figure 3E). *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Fusobacteria* were major phyla determinants for sample differentiation.

More detailed analysis revealed that DNA and RNA samples differed from each other by 12, 10, and 30 phylotypes and by 18, 17, and 35 genera in the tumor, tumor adjacent, and control groups, respectively (Table 1, Supplementary Table 4). In all study groups, bacteria such as *Neisseria*, *Peptostreptococcus*, *Prevotella*, *Veillonella*, and *Oribacterium* were significantly more abundant at the DNA level, while *Staphylococcus*, *Methyloversatilis*, *Pseudomonas*, *Reyranella*, *Corynebacterium*, and *Sediminibacterium* were significantly enriched at the RNA level. Interestingly, most of these bacteria founded in the RNA samples were not observed in the DNA samples at all, or their relative abundance was low. Some

Table 1 List of significantly (*P* value < 0.05) different genera between DNA and RNA samples in the control, tumor, and tumor adjacent groups

	Increased or decreased at DNA level		
	H	T	Tadj
H & T & Tadj			
Haemophilus	Increased	Decreased	Increased
Methyloversatilis	Decreased	Decreased	Decreased
Neisseria	Increased	Increased	Increased
Peptostreptococcus	Increased	Increased	Increased
Prevotella	Increased	Increased	Increased
Pseudomonas	Decreased	Decreased	Decreased
Reyranella	Decreased	Decreased	Decreased
Sediminibacterium	Decreased	Decreased	Decreased
Staphylococcus	Decreased	Decreased	Decreased
Veillonella	Increased	Increased	Increased
H			
Actinomyces	Increased		
Gemella	Decreased		
Helicobacter	Decreased		
Streptococcus	Decreased		
T & Tadj			
Fusobacterium		Increased	Increased
Granulicatella		Increased	Increased
Porphyromonas		Increased	Increased
Solobacterium		Increased	Increased

The table presents only those bacteria genera, the mean relative abundance of which at least in one of the compared groups exceeded 1%.

changes in the relative abundance of bacteria between DNA and RNA samples were specific for the study group. For instance, in the control group, *Helicobacter*, *Gemella*, and *Streptococcus* were enriched at the RNA level, while *Actinomyces* and *Alloprevotella* were enriched at the DNA level. In the GC groups (tumor and tumor adjacent), *Fusobacterium*, *Granulicatella*, *Solobacterium*, and *Porphyromonas* were enriched at the DNA level. No bacteria were enriched at the RNA level in this group.

Nevertheless, despite the found differences between DNA and RNA, samples of the same origin tended to cluster together in each of the study groups (Figure 2). Paired samples, 46 pairs out of 64 (72%) in the tumor group and 38 pairs out of 58 (66%) in the tumor adjacent tissue group, clustered next to each other, indicating their global similarity (Supplementary Figure 1). Paired samples from the control group were not added to this analysis due to the small number of paired samples.

Revealed microbiome alterations in GC depend on the chosen sequencing modality

The GC samples had lower bacterial richness and diversity compared to control samples (Figures 3F and 3G). While differences in diversity were found both at the DNA and RNA levels, differences in bacterial richness were found only at the RNA level. Group-average agglomerative hierarchical clustering analysis showed that it was possible to distinguish patients with GC from controls by their bacterial profile, as samples tended to cluster based on clinical status (both at the DNA and RNA levels) (Figures 2B and 2C). These results were supported by the phylogenetic analysis of global stomach bacteria, which revealed significant differences between the GC group and control groups at all taxonomic ranks (Figures 3A-C, Supplementary Table 3).

Bacterial abundance differential analysis revealed 15 phylotypes and 17 genera that differed between the GC and control groups at the DNA level (Table 2, Supplementary Figure 2, Supplementary Table 4). Meanwhile, at the RNA level, there were twice as many differences: 40 at the phylotype level and 27 at the genus level. Half of the differences detected at the DNA level were also found at the RNA level (58%

Table 2 List of significantly (*P* value < 0.05) different genera between the control and gastric cancer groups, depending on the selected study material

T *vs* H	Increased or decreased in T		Tadj *vs* H	Increased or decreased in Tadj	
	DNA	RNA		DNA	RNA
Lactobacillus	Increased	Increased	*Lactobacillus*	Increased	Increased
Actinomyces	Decreased	Decreased	*Actinomyces*	Decreased	Decreased
Atopobium	Decreased	Decreased	*Atopobium*	Decreased	Decreased
Granulicatella	Decreased	Decreased	*Granulicatella*	Decreased	Decreased
Propionibacterium	Decreased	Decreased	*Propionibacterium*	Decreased	Decreased
Streptococcus	Decreased	Decreased	*Streptococcus*	Decreased	Decreased
Veillonella	Decreased	Decreased	*Veillonella*	Decreased	Decreased
Rothia	Decreased	Increased	*Rothia*	Decreased	Decreased
Clostridium sensu stricto	Increased		*Leptotrichia*	Decreased	
Prevotella	Decreased		*Prevotella*	Decreased	
Pseudomonas		Increased	*Pseudomonas*		Increased
Staphylococcus		Increased	*Staphylococcus*		Decreased
Gemella		Decreased	*Gemella*		Decreased
Methyloversatilis		Decreased	*Methyloversatilis*		Decreased
Parvimonas		Decreased	*Parvimonas*		Decreased
Reyranella		Decreased	*Reyranella*		Decreased
Sediminibacterium		Decreased	*Sediminibacterium*		Decreased

The table presents only those bacteria genera, the mean relative abundance of which at least in one of the compared groups exceeded 1%.

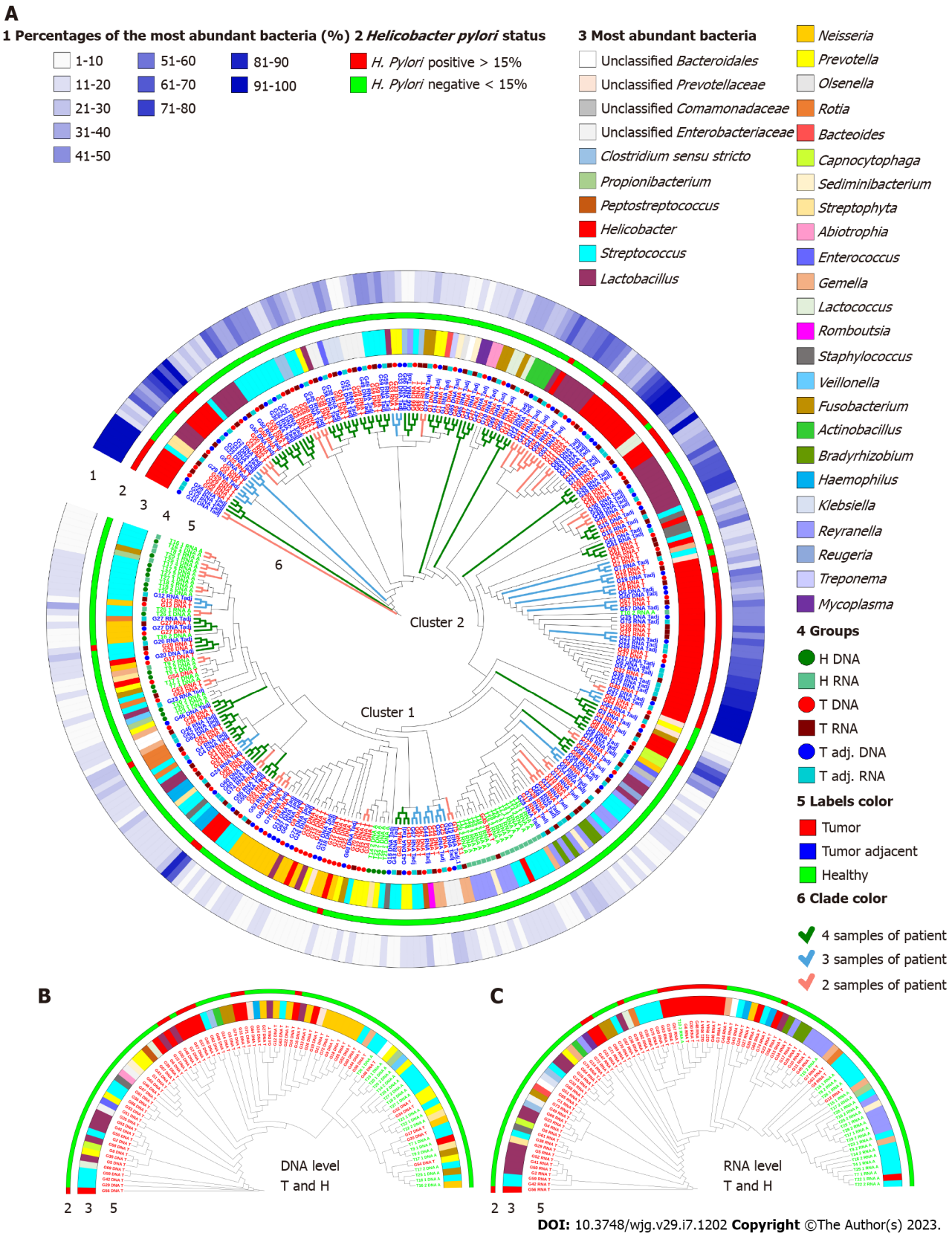
of genera and 46% of phylotypes). These bacteria include previously described bacteria, such as *Lactobacillus, Propionibacterium, Streptococcus,* and *Veillonella,* among others[25-29].

Although fewer unique bacteria were identified only at the DNA level (8 phylotypes and 7 genera), they were more studied and more frequently discussed in the literature as being associated with various human health conditions (Supplementary Table 4). These bacteria include *Campylobacter*, *Clostridium sensu stricto*, *Prevotella*, and *Saccharibacteria,* among others. Of the listed bacteria, *Clostridium sensu stricto* was enriched, and others were decreased in GC patients. Uniquely, only at the RNA level were 33 and 17 differences at the phylotype and genus levels, respectively, found between the GC and control groups (Supplementary Table 4). Essentially, this group included such bacteria that were not established or their abundance at the DNA level was negligible, for example, *Limnohabitans*, *Methylobacterium*, *Methyloversatilis*, *Pseudomonas*, *Reyranella*, *Rhodoluna*, *Sediminibacterium*, and *Staphylococcus*. Of the listed bacteria, only *Pseudomonas* and *Staphylococcus* were more abundant in GC samples, while all the others were more abundant in healthy individuals.

Bacterial diversity and profile comparison analysis between tumor and tumor adjacent tissues did not reveal significant differences at either the DNA or RNA level (Figures 3A-C, 3F and 3G). Moreover, assemblages of approach from each individual typically clustered together irrespective of tissue type (tumor or tumor adjacent tissue) (Figure 2A).

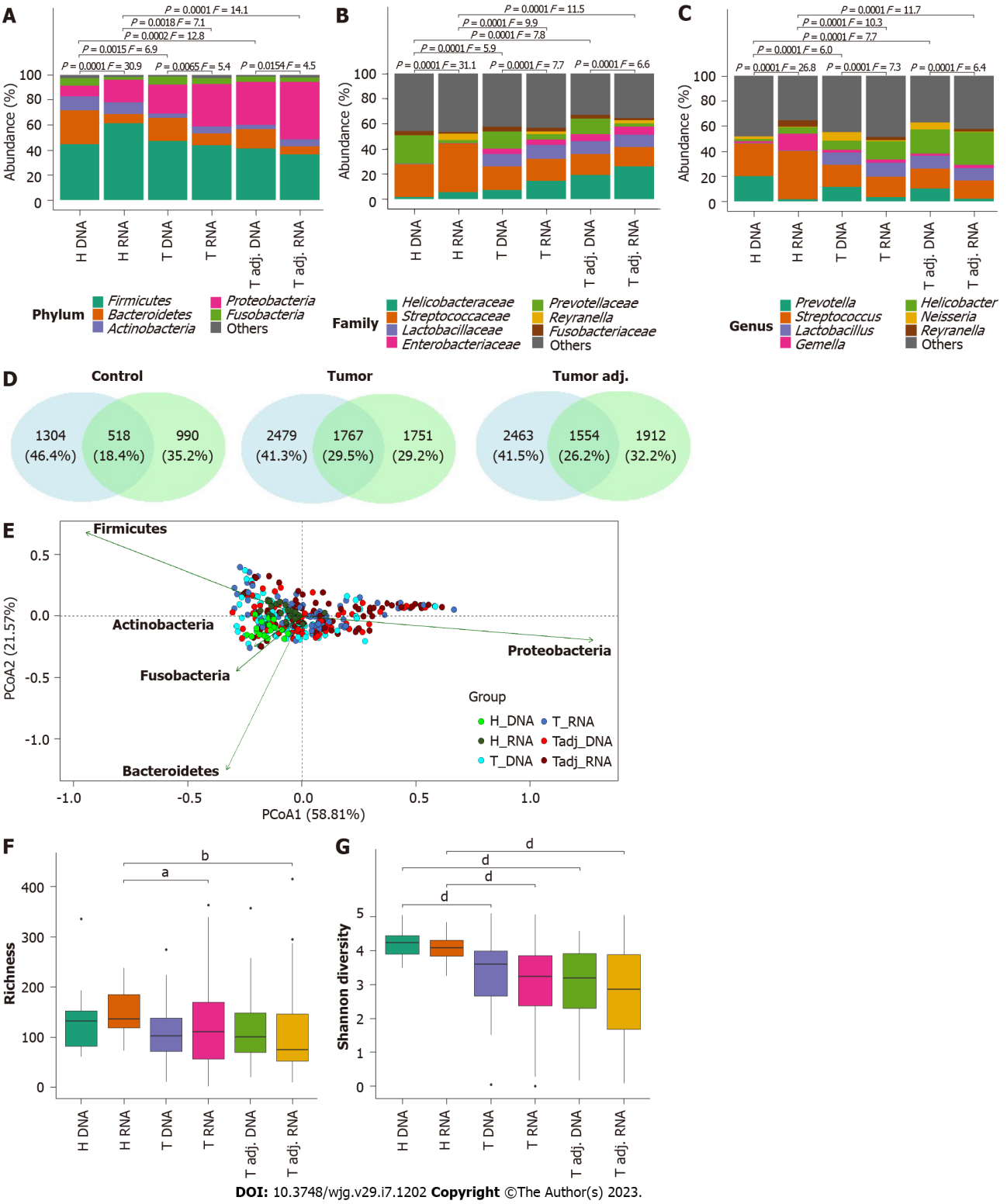
Bacterial networks in GC patients have fewer components and integrated connections

Analysis of the bacterial network similarity revealed that the main network holding bacteria with the highest betweenness centrality score was different between DNA and RNA levels in all study groups (Supplementary Figure 3). In the bacterial network of the control group at the DNA level, phylotypes depending on the *Streptococcus, Prevotella,* and *Actinomyces* genera accounted for 68% (58 out of 85) of the total number of bacteria and formed the core network keeping bacteria, while at the RNA level, core bacteria were *Streptococcus* and *Gemella*, making up to 62% (47 out of 75) (Supplementary Figures 3A and 3B). The GC groups showed different DNA/RNA networks as well: The main network forming bacteria in the tumor adjacent tissue at the DNA level was *Prevotella*, *Gemella*, and *Granulicatella*, while the RNA network was shaped by *Streptococcus, Reyranella,* and *Fusobacterium* (Supplementary Figures 3C and 3D). The most critical network-forming bacteria in tumor tissue were: *Granulicatella*, *Veillonella*, and *Neisseria* at the DNA level and *Reyranella*, *Acinetobacter*, and *Prevotellaceae* at the RNA level (



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Figure 2 Group-average agglomerative hierarchical clustering at the phylotype level based on global bacterial profiles. A: Clustering of 139 gastric cancer (GC) tumor, 134 tumors adjacent and 43 control group samples according to individual and general bacterial profiles; B and C: Clustering of GC tumor tissues and the control group at the DNA and RNA levels. The first circle (1) depicts the part of the most abundant bacteria; the second circle (2) represents the status of *Helicobacter pylori*; the third circle (3) shows the most abundant bacteria at the genus level; fourth and fifth circles (4 and 5) display the samples belonging to a certain study group; clade colors (6) represent the abundance of each patient sample clustered together. *H. pylori*: *Helicobacter pylori*.



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Figure 3 Shift and diversity of stomach microbiota of healthy controls, gastric cancer patient's tumor and tumor adjacent tissues depending on microbial source (DNA and RNA). A: Relative mean abundance of the top 5 microbiota at the phylum; B: Top 7 microbiota at the family; C: Top 7 microbiota at the genus level. Formal comparisons between groups were evaluated by analysis of similarity (PERMANOVA); D: Venn diagrams show the number of common and group-specific phylotypes comparing each study group at the DNA and RNA levels; E: Principal coordinate analysis of bacterial beta diversity of all study groups at the phylum level; F and G: Comparison of bacterial alpha diversity: Bacterial richness and bacterial diversity. $^{a}q < 0.05$, $^{b}q < 0.01$, $^{d}q < 0.0001$.

Supplementary Figures 3E and 3F).

Two common bacterial clusters (one at the DNA level and another at the RNA level) with strong positive correlations for tumor and tumor adjacent tissues were discovered (Supplementary Figures 3C-F), which confirms the absence of significant differences between tumor and tumor adjacent tissue microbiome profiles. At the DNA level, the common cluster consisted of Phy6 (*Neisseria*), Phy15 (unclas-

sified *Prevotellaceae*), Phy23 (*Neisseria perflava*), Phy29 (*Prevotella melaninogenica*), Phy87 (*Solobacterium*), and Phy98 (*Prevotella*). The common cluster at the RNA level included phylotypes such as Phy7 (*Reyranella*), Phy33 (*Sediminibacterium*), Phy46 (*Propionibacterium acnes*), Phy94 (*Methyloversatilis*), Phy107 (*Pseudomonas aeruginosa*), and Phy108 (*Sphingomonas echinoides*). Detected clusters were not found in the control group.

Generally, under the same analysis conditions, GC patients displayed a simpler bacterial network at both the DNA and RNA levels. At the DNA level, control, the tumor, and tumor adjacent groups had 85, 25, and 23 bacteria, respectively; at the RNA level, they had 75, 21, and 18, respectively. Moreover, analysis of bacterial interactions in controls had not only positive but also negative correlations, while GC analysis showed mostly positive correlations.

GC microbiota alterations and clinical parameters

At the DNA level, according to clinical parameters, statistically significant differences were found only in the decrease in bacterial richness between smaller tumors (T1-T2) and extended tumors (T4). The RNA level turned out to be more sensitive and allowed us to detect richness differences between grade II and grade III (Figures 4A and 4B). Moreover, at the RNA level, the relative abundance of the Phy7 (*Reyranella*) and Phy33 (*Sediminibacterium*) phylotypes was lower in the diffuse type of GC than in the intestinal type (Figures 4C and 4D). No differences were found between subgroups at the DNA level.

The effect of H. pylori infection on stomach microbiota

PCoA showed *Helicobacter* to be the major determinant for differentiating samples based on their bacterial composition in the stomach (Supplementary Figure 4). Overall, *H. pylori* was detected in 115 and 117 DNA and RNA samples, respectively. In the control group, *H. pylori* was lower than that in the GC groups at both the DNA and RNA levels (Figure 5A). The tumor adjacent sample group showed the highest number of samples with high *H. pylori* abundance (Figure 5B). Both in tumor and tumor adjacent groups the mean abundance of *H. pylori* was increased at the RNA level, although no significant differences between DNA and RNA samples were found.

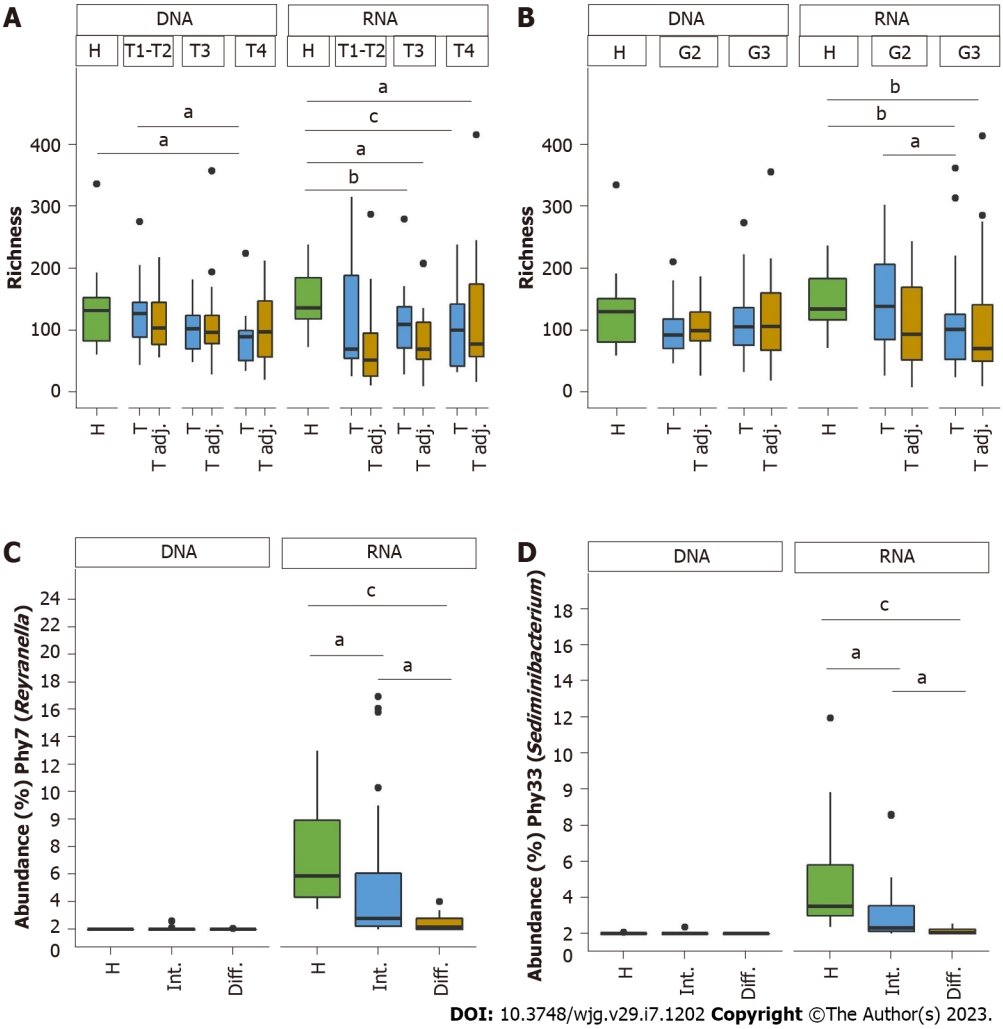
High *H. pylori* relative abundance (> 15%) led to an increase in the relative abundance of *Proteobacteria* and a decrease in other major bacterial phyla, such as *Firmicutes*, *Bacteroidetes*, and *Fusobacteria* (Figure 5C). In tumor tissues analyzed at the DNA and RNA level, *Helicobacter* was only one genus which changed significantly between samples with high and low *H. pylori* abundance (Figure 5D, Supplementary Table 5). On the other hand, in tumor adjacent tissues, more bacteria were found, the number of which changed together with *Helicobacter*. At the DNA level, as the relative abundance of *H. pylori* increased, the abundance of *Porphyromonas* and *Prevotella* significantly decreased. In line with our previous results, more significant differences were found at the RNA level: *Staphylococcus* significantly increased and seven bacteria (*Campylobacter*, *Fusobacterium*, *Prevotella*, *Pseudomonas*, *Reyranella*, *Sediminibacterium*, *Streptococcus*) decreased (Figure 5C, Supplementary Table 5). *Porphyromonas* tended to decrease in tumor adjacent RNA samples as well, although it did not reach a statistically significant level (Supplementary Table 5).

DISCUSSION

Despite growing interest in the study of microbiota, there is still limited agreement on the most appropriate standard for such studies, especially using 16S rRNA sequencing. Here, we performed systematic analysis of bacterial communities at both the 16S rRNA gene and 16S rRNA transcript levels. To estimate the impact of the different approaches, we used the GC model and considered not only healthy gastric tissues but also GC tumor and adjacent tissues.

The analysis of the study results showed that there were significant differences in the relative abundance of the gastric tissue microbiome between 16S rRNA gene transcript and 16S rRNA gene levels in all study groups (control, tumor, and tumor adjacent). This is the first GC study indicating that active and standing gastric microbiomes are distinct even at the largest taxonomic levels.

Differences in bacterial communities at the DNA and RNA levels could be explained by several possibilities. Using DNA as a research material summed up all bacteria, both biologically active passive in the form of endospores, and DNA sequences of already destroyed and dead bacteria[2,30]. The presence and number of ribosomes in bacteria reflects their metabolic activity; thus, the analysis at the RNA level shows the metabolic activity of live and active bacteria in the community[31-33]. For instance, previously GC-associated bacteria such as *Prevotella*, *Veillonella*, and *Neisseria* in our study were present in high abundance in all analyzed groups at the DNA level but were greatly reduced at the RNA level. In contrast, *Pseudomonas*, *Reyranella*, and *Staphylococcus* were present in higher abundance at the RNA level in all groups. However, it is erroneous to assume that only active bacteria can influence host responses. Many studies have shown that inactivated bacteria or parts of their cells can also influence inflammatory processes or other responses in host tissues. For example, Rabie *et al*[34] showed that thermally inactivated *Salmonella*, *Staphylococcus*, *Escherichia*, and *Pseudomonas* strains with unchangeable surface proteins cause colon and breast cancer cell proliferation. In Suprewicz *et al*[35]'s



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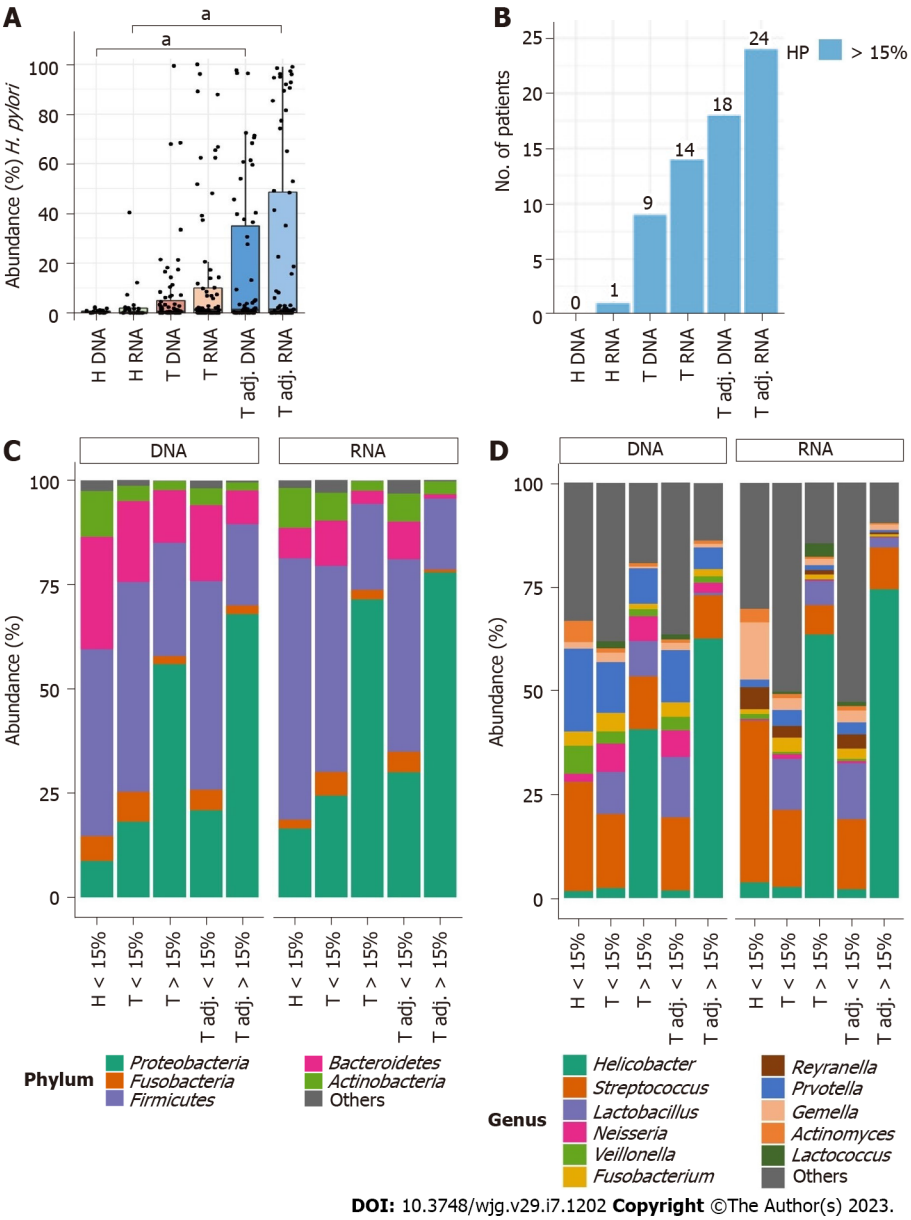
Figure 4 Comparison of bacterial abundance and richness in relation to clinical data at the DNA and RNA levels. A: Bacterial richness according to tumor size; B: Bacterial richness according to grading; C and D: Comparison of the relative mean abundance of Phy7 (*Reyranella*) and Phy33 (*Sediminibacterium*). Statistically significant differences between study groups are indicated, $^{a}q < 0.05$, $^{b}q < 0.01$, $^{c}q < 0.001$.

study, heat-inactivated *Enterococcus faecalis*, *Actinomyces odontolyticus*, and *Propionibacterium acnes* caused cell proliferation changes in lung, breast, and ovarian carcinoma. Postbiotics work based on the same principle. To avoid possible bacterial infection during therapy, instead of active bacteria, their metabolites, which are involved in anti-inflammatory and anticancer mechanisms, are used[36].

It is also cannot be excluded that the shift in bacterial abundance between DNA and RNA levels might stem from varying numbers of copies of the 16S rRNA gene[37] or target sequence quantity inequality[38]. Bacterial rRNAs (16S rRNA, 23S rRNA and 5S rRNA) are typically organized into one operon, and their transcription occurs together, with the number of such operons varying from 1 to 15 [38]. In the case of active bacteria, an increase in 16S rRNA gene copies proportionally increases the pool of 16S rRNA transcripts. However, in the case of inactive bacteria, a larger number of 16S gene copies enables the detection of some bacteria, which could not be detected at the RNA level.

The amount of target sequences using the 16S rRNA gene and its transcript are not the same. Of all types of RNA molecules present in the cell, the most common (80%-90%) are included in the ribosome structure rRNAs[39]. 16S rRNAs make up one-third of the total rRNAs. On the other hand, when analyzing the microbiota using the 16S rRNA gene, only one gene is amplified out of the total number of genes, which in different bacteria varies from 1500 to 7000[37]. Thus, the initial larger amount of the bacterial target sequence at the RNA level makes it possible to increase the depth of sequencing and detect more rare bacteria that would be lost during DNA-level analysis. In addition, a shift toward DNA or RNA levels can also be caused by ingestion of bacterial parts from the higher parts of the digestive tract.

Our analysis revealed that the profile of differences found between GC and control tissue depended on the chosen modality: At the DNA level, 17 bacterial genera were detected, and at the RNA level, 27 bacterial genera were detected. Ten of those bacteria (*Actinomyces*, *Alloprevotella*, *Atopobium*, *Granulicatella*, *Lactobacillus*, *Megasphaera*, *Propionibacterium*, *Rothia*, *Streptococcus*, *Veillonella*) were found to be different from the control group at both levels of sequencing; seven bacterial taxa (*Campylobacter*,



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Figure 5 Comparison of gastric microbiota alterations at the DNA and RNA levels depending on high *Helicobacter pylori* abundance (> 15%). A and B: Relative mean abundance of *Helicobacter pylori* (*H. pylori*) in all study groups and the number of patients with high *H. pylori* abundance; C and D: Comparison of the relative abundance of the top 5 microbiota at the phylum and top 11 microbiota at the genus levels. [a]$q < 0.05$.

Clostridium sensu stricto, *Leptotrichia*, *Oribacterium*, *Prevotella*, *Saccharibacteria genera incertae sedis*, *Stomatobaculum*) were found uniquely only at the DNA level; and 17 (*Anaerococcus*, *Corynebacterium*, *Eubacterium* , *Flavobacterium*, *Gemella*, *Legionella*, *Limnohabitans*, *Massilia*, *Methylobacterium*, *Methyloversatilis*, *Parvimonas*, *Pseudomonas*, *Reyranella*, *Rhodoluna*, *Sediminibacterium*, *Solobacterium*, *Staphylococcus*) were found uniquely only at the RNA level. These results confirm the importance of unifying the procedures for studying the microbiota.

Although our study focused on differences in methodology, it did reveal several important findings for the GC study as well. Fourteen bacteria genera were identified to be decreased in patients with GC. Eleven of these bacteria (*Actinomyces*, *Atopobium*, *Propionibacterium*, *Streptococcus*, *Granulicatella*, *Veillonella*, *Rothia*, *Parvimonas*, *Gemella*, *Prevotella*, *Leptotrichia*) were previously established in the stomach of healthy people in the absence of gastrointestinal diseases[40]. Most of them are common members of the upper gastrointestinal tract and have strong enzymatic activities. Our study also found four bacteria genera, which were significantly increased in GC patients' stomach biopsy: *Lactobacillus*, *Clostridium sensu stricto*, *Staphylococcus*, and *Pseudomonas*.

Lactobacillus is commonly used as a probiotic; however, it has been verified in multiple studies to be enriched in GC[41]. *Lactobacillus* strains, as well as *Clostridium* and *Staphylococcus*, can reduce nitrate to nitrite[42,43]. During the nitrate-reducing process, many N-nitroso compounds are formed that inhibit cell apoptosis and promote mutagenesis and protooncogene expression[44-47]. *Clostridium* is part of the

normal gastrointestinal tract; however, in several previous studies, as in ours, an increase in the number of *Clostridium sensu stricto* was found[48-50]. Interestingly, Lertpiriyapong *et al*[9] showed earlier onset and faster progression of GC in INS-GAS mice with restricted microbiota (including *Clostridium*, *Lactobacillus,* and *Bacteroides*), highlighting a possible role of these bacteria in GC. Additionally, several studies have detected increased levels of *Staphylococcus* in patients with upper gastrointestinal diseases[51-53]. One of the reasons for this may be that stains of *Staphylococcus* have the enzyme urease, and are able to catalyze the hydrolysis of urea to carbon dioxide and ammonia, which can neutralize gastric hydrochloric acid, thus promoting bacterial existence. Although we found an increased number of *Pseudomonas*, this bacterial infection affects people with weakened immune systems (including patients with cancer), and thus, it is more likely that this finding is the result of already developed pathological processes.

We did not detect significant bacterial abundance, richness, or diversity alterations at either the DNA or RNA level between tumor and tumor adjacent tissues. This result is consistent with two previous studies[54,55] but contradicts recent GC studies where significant differences between tumor-affected and nearby healthy tissues were found[27,56]. Moreover, we found the same clusters of bacterial networks in tumor and tumor adjacent tissues at both the DNA and RNA levels. These results may suggest that with the onset and development of carcinogenic processes, local changes in stomach tissues lead not only to a change in the bacterial composition but are also precise uniformity between cancer-affected and still healthy tissues (at least within a radius of 5 cm from the tumor area).

Studying GC samples at the RNA level, we managed to identify microbiome associations with clinical data. Analysis revealed two phylotypes (Phy7 and Phy33) related to *Reyranella* and *Sediminibacterium*, respectively. The relative number of those phylotypes gradually decreased from healthy to GC patients through intestinal growth type (considered as less aggressive cell growing type) to GC patients with diffuse growth type of cancer cells with worse outcome prognosis. To our knowledge, this is the first mention of these bacteria associated with the GC cell growth type. *Reyranella* is part of *Proteobacteria* and has previously been associated with the main chemokine expression, which is involved in T-cell attraction during cancerogenesis[57,58]. In another study, it was shown that there are significantly lower amounts of circulating natural killer and Treg cells in patients with diffuse/mixed-type GC compared to intestinal-type GC[59]. Taken together, these results suggest that *Reyranella* may be involved in the decrease in T-cell number and thus stimulation of cell growth of diffuse-type GC. *Sediminibacterium* was reported to be associated with GC, but there is no knowledge about the possible role of this bacteria in the pathophysiological processes[60,61]. Therefore, more detailed research on the effects of *Reyranella* and *Sediminibacterium* on GC cells is needed to be able to use these bacterial phylotypes as potential biomarkers.

H. pylori is the most common bacterial infection worldwide, as well as the main risk factor for GC [40]. It has been shown that during the transition from *H. pylori*-induced inflammation to the growth and development of carcinogenic cells, *H. pylori* is no longer detected in the affected areas in such large abundance[62]. Our results, showing that more *H. pylori* were found in tumor adjacent tissue than in tumor tissue, both at the DNA and RNA levels, confirm this. According to some previous reports, infection with *H. pylori* promotes the proliferation of non-*Helicobacter* bacteria from *Proteobacteria*, *Spirochetes*, and *Acidobacteria* and limits the spread of bacteria such as *Actinobacteria*, *Bacteroidetes* and *Firmicutes*[63,64]. Although most of the bacteria we found with altered numbers in GC were not associated with *H. pylori*, changes in the number of bacteria, such as *Granulicatella*, *Lactobacillus*, *Rothia*, *Pseudomonas*, *Gemella*, *Prevotella*, *Leptotrichia*, *Clostridium sensu stricto*, and *Fusobacterium,* were associated with high *H. pylori* abundance.

The question regarding the causality in the gastric microbiome is still partially unanswered. On the one hand, alterations in gastric microbiota have a causal role in the progression of carcinogenesis (*e.g.*, *H. pylori*). On the other hand, the role of other bacteria is less understood. However, there are new studies that strongly suggest the impact of the gastric microbiome on inflammation and carcinogenesis. For instance, a recent study by Kwon *et al*[65] showed that intestinal metaplasia or GC patient gastric microbiome transplantation contributes to changes in the phenotype of premalignant lesions. In this regard, a detailed understanding of the output of different sequencing technologies and comparability between RNA/DNA-based analyses is critical.

Since systematic analysis to assess the differences with respect to GC has not been performed before, we would like to point out some limitations of this work. While the primary focus of the work was related to technical differences, thus food preferences, sex, and aging can be potential contributing factors that have not been thoroughly considered in this study. Overall, the focus was on providing a truly confirmed healthy cohort for the most precise comparison to strengthen the differences. Nevertheless, PERMANOVA and Mann-Whitney analyses performed in each of the study groups (tumor DNA, tumor RNA, tumor adjacent DNA, tumor adjacent RNA, control DNA, control RNA) did not reveal significant differences between the sexes and age (divided by median) (Supplementary Tables 6 and 7). Furthermore, due to the sample size, we did not consider the newly proposed TCGA classification for subsequent analysis nor to assess the impact of 4 subtypes on bacterial composition in tumors.

CONCLUSION

In conclusion, our study provides evidence that the tumor microbiome of GC patients has a distinct pattern compared to healthy controls, while the difference analyzed from adjacent tissue was rather low. Despite some overlap between the data obtained from the 16S rRNA transcript and 16S rRNA gene, our results showed the critical importance of the chosen study material on the resulting bacterial profile. Thus, researchers comparing their results with previous studies might take into consideration which initial material was used, either the 16S rRNA gene or 16S rRNA transcript. Our results showed that the RNA level was more sensitive for detecting low abundance bacteria and allowed us to detect differences according to GC clinical data.

ARTICLE HIGHLIGHTS

Research background

There is currently no gold standard for analyzing the microbiome in 16S rRNA studies. Two common modalities are: Sequencing of DNA (16S rRNA gene) and sequencing of RNA (16S rRNA transcript). Gastric cancer (GC) remains one of the most common cancers in the world and microbiome takes important place in its carcinogenesis.

Research motivation

Microbiota studies are becoming more relevant and widespread. Comparison of different approaches for microbiome studying is necessary for correct interpretation of other studies results, as well as for a deeper understanding of bacterial composition.

Research objectives

To investigate how the choice of sequencing modality affects the bacterial profile of differences between case and controls as well as to characterize the microbiota of GC tissues using 16S rRNA gene and its transcript.

Research methods

The study included healthy tissues from the control group, as well as tumor and tumor adjacent tissues from GC patients. From all biopsies RNA and DNA were extracted. 16S rRNA V1-V2 region was sequenced for all samples. For significant differences between groups permutational multivariate analysis of variance and Mann-Whitney test followed by false-discovery rate test were used.

Research results

Only a small portion of bacterial sequences overlapped on DNA and RNA levels in all groups. Differences between GC and control groups also only partially overlayed on DNA and RNA levels. RNA sequencing was more sensitive for detecting differences in bacterial richness, low abundance bacteria, and changes in the relative abundance of *Reyranella* and *Sediminibacterium* according to the type of GC. In each study group differences between DNA and RNA bacterial profiles were identified.

Research conclusions

Chosen study material (16S rRNA transcript or 16S rRNA gene) greatly affects detectable microbiome profile as well as the differences between cases and controls.

Research perspectives

This study provides microbiome analysis applying two different methodologies using GC gastric tissues as example and could serve as a reference for future research.

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FOOTNOTES

Author contributions: Vilchez-Vargas R, Link A, Lehr K, Kupcinskas J, Skieceviciene J and Nikitina D contributed to the study design, analysis, and interpretation of the data; Nikitina D, Link A, and Skieceviciene J drafted the manuscript; Skieceviciene J, Kupcinskas J, Vilchez-Vargas R and Link A supervised the study procedures and revised the manuscript; Nikitina D, Lehr K, and Vilchez-Vargas R performed bioinformatic and statistical analyses; Urba M and Jonaitis LV obtained participant's samples and provided clinical data; Skieceviciene J and Link A have contributed equally to this work and share last authorship; and all authors read and approved the final version of the manuscript.

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Country/Territory of origin: Lithuania

ORCID number: Darja Nikitina 0000-0002-9942-5118; Konrad Lehr 0000-0003-3213-8138; Ramiro Vilchez-Vargas 0000-0001-6535-8212; Laimas Virginijus Jonaitis 0000-0001-9086-3675; Mindaugas Urba 0000-0003-0910-9072; Juozas Kupcinskas 0000-0002-8760-7416; Jurgita Skieceviciene 0000-0002-4893-6612; Alexander Link 0000-0002-9514-4562.

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SYSTEMATIC REVIEWS

Influence of methyl donor nutrients as epigenetic regulators in colorectal cancer: A systematic review of observational studies

Lourdes Pilar Chávez-Hidalgo, Silvia Martín-Fernández-de-Labastida, Marian M de Pancorbo, Marta Arroyo-Izaga

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Lourdes Pilar Chávez-Hidalgo, Silvia Martín-Fernández-de-Labastida, Marta Arroyo-Izaga, Department of Pharmacy and Food Sciences, Faculty of Pharmacy, University of the Basque Country UPV/EHU, Vitoria-Gasteiz 01006, Araba/Álava, Spain

Marian M de Pancorbo, Department of Z. and Cellular Biology A., University of the Basque Country UPV/EHU, Vitoria-Gasteiz 01006, Araba/Álava, Spain

Marian M de Pancorbo, Marta Arroyo-Izaga, BIOMICs Research Group, MICROFLUIDICs and BIOMICs Cluster UPV/EHU, Lascaray Research Center, University of the Basque Country UPV/EHU, Vitoria-Gasteiz 01006, Araba/Álava, Spain

Corresponding author: Marta Arroyo-Izaga, PharmD, Department of Pharmacy and Food Sciences, Faculty of Pharmacy, University of the Basque Country UPV/EHU, Paseo de la Universidad, No. 7, Vitoria-Gasteiz 01006, Araba/Álava, Spain. marta.arroyo@ehu.eus

Abstract

BACKGROUND

Dietary methyl donors might influence DNA methylation during carcinogenesis of colorectal cancer (CRC). However, whether the influence of methyl donor intake is modified by polymorphisms in such epigenetic regulators is still unclear.

AIM

To improve the current understanding of the molecular basis of CRC.

METHODS

A literature search in the Medline database, *Reference Citation Analysis* (https://www.referencecitationanalysis.com/), and manual reference screening were performed to identify observational studies published from inception to May 2022.

RESULTS

A total of fourteen case-control studies and five cohort studies were identified. These studies included information on dietary methyl donors, dietary components that potentially modulate the bioavailability of methyl groups, genetic variants of methyl metabolizing enzymes, and/or markers of CpG island methylator phenotype and/or microsatellite instability, and their possible interactions on CRC risk.

CONCLUSION
Several studies have suggested interactions between methylenetetrahydrofolate reductase polymorphisms, methyl donor nutrients (such as folate) and alcohol on CRC risk. Moreover, vitamin B_6, niacin, and alcohol may affect CRC risk through not only genetic but also epigenetic regulation. Identification of specific mechanisms in these interactions associated with CRC may assist in developing targeted prevention strategies for individuals at the highest risk of developing CRC.

Key Words: Colorectal cancer; DNA methylation; Epigenetics; Methyl donors; Microsatellite instability; Nutrients

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Core Tip: Dietary methyl donors might influence DNA methylation during the carcinogenesis of colorectal cancer (CRC). However, whether the influence of methyl donor intake is modified by polymorphisms in such epigenetic regulators is still unclear. We conducted a systematic review on this topic to improve the current understanding of the molecular basis of CRC. Several studies have suggested interactions between methylenetetrahydrofolate reductase polymorphisms, methyl donor nutrients (such as folate) and alcohol on CRC risk. Moreover, vitamin B_6, niacin, and alcohol may affect CRC risk through not only genetic but also epigenetic regulation.

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INTRODUCTION

Colorectal cancer (CRC) is the third most frequent type of cancer and is responsible for the second highest mortality rate in cancer patients worldwide[1]. Although screening for early detection of CRC is effective to help decrease the trends in mortality rates, understanding daily life factors is also important to prevent this type of cancer[2]. The main factors which may help prevent CRC are those associated with diet, lifestyle, and prevention of metabolic diseases[3].

With regards to the dietary component, nutrients associated with one carbon (1C) metabolism [including folate, other B vitamins, methionine (Met), and choline] have been recognized as anticarcinogenic and chemotherapeutic agents in the 1C metabolic network[4]. Folate has shown to play a preventive role in CRC, probably because of its involvement in the processes of DNA methylation and synthesis[5]. Other nutrients, such as Met and vitamins B_6 and B_{12}, which interact metabolically with folate in this process, may also influence the risk of CRC[6]. Moreover, in some of those studies, the observed inverse association between folate status and CRC risk was further modified by genetic polymorphisms of the enzymes involved in folate metabolism, most notably methylenetetrahydrofolate reductase (*MTHFR*).

A common C677T substitution in the *MTHFR* gene results in a protein with valine instead of alanine, yielding a more thermolabile enzyme with decreased activity[7]. Numerous studies have shown that this variant (TT) is associated with a decreased risk of CRC, but only when folate status is normal or high. *MTHFR* polymorphism is possibly the best-known gene polymorphism that switches from being a risk factor to a protective one depending on nutrient status. In any case, it is also important to evaluate the joint influence that other polymorphisms in genes involved in folate metabolism might exert. Thus, for example, Met synthase requires vitamin B_{12}, as methylcobalamin, as a cofactor. A variant in this gene, A2756G [in methionine synthase (*MTR*) gene], has been described and results in the substitution of aspartate by glycine. Some studies have shown that the MTR 2756 GG genotype is associated with a decreased risk of CRC; however, the association with diet is still unclear[8,9].

Another relevant example of a mutation in a polymorphism associated with CRC risk, but not consistent with nutrients, is the case of serine hydroxymethyltransferase, a pyridoxal phosphate (B_6)-dependent enzyme, in particular the polymorphism C1420T[10]. An additional relationship between 1C metabolism-related nutrients and CRC risk is related to its influence on DNA methylation. Thus, low folate status or intake is related to a decreasing methylation level[11,12], whereas colonic mucosal DNA methylation increased globally as a result of folate supplementation[13]. A sufficient intake of methyl

donors may also prevent aberrant CpG island promoter hypermethylation. The promoter CpG island hypermethylation that characterizes the CpG island methylator phenotype (CIMP) in CRC is common [14]. Moreover, polymorphisms in enzymes involved in folate metabolism may change the potential impact of methyl donor consumption on DNA methylation[15].

According to the molecular subtype, chromosomal instability, and microsatellite instability (MSI) represent the major pathways for CRC[16]. The inactivation of DNA mismatch repair (MMR) genes, which are responsible for correcting mismatched bases during DNA replication, results in MSI[17]. Microsatellites are short sequences that are dispersed across the genome and are likely to undergo MMR machinery-induced deletion or insertion. Defects in the MMR machinery are associated with CRC[18] and can be affected by epigenetic alterations and deregulation of methylation[19]. In this sense, the associations between methyl donor nutrient intake and CRC risk have been extensively studied, although evidence on their effect is limited[20]. However, whether they act as effect modifiers against a background of deficient DNA repair capacity is unknown.

Until now, there have been few studies on the influence of methyl group donors as epigenetic regulators in CRC[21]. In the present work, we reviewed previous studies that have investigated this matter to improve the current understanding of the molecular basis of CRC, which could contribute to a better design of future research and to better preventive nutritional management in this type of cancer.

MATERIALS AND METHODS

Search strategy

The search terms and search strategy were developed by two researchers. To guide this research, we formulated the following question as the starting point: What is the influence of methyl donor nutrients as epigenetic regulators in CRC? For this purpose, a systematic search in the Medline (through PubMed) database, *Reference Citation Analysis* (https://www.referencecitationanalysis.com/, an artificial intelligence technology-based open multidisciplinary citation analysis database), and a manual reference screening were performed to identify observational studies published from inception to May 20, 2022. A search for relevant keywords and medical subject heading terms related to dietary methyl donors, dietary components that potentially modulate the bioavailability of methyl groups, genetic variants of methyl-metabolizing enzymes, markers of CIMP and/or MSI, in combination with keywords related to CRC events was conducted.

The search was amplified through citation chaining (forward and backward) of the included studies. Reference lists of all identified articles and other related review articles, systematic reviews, and meta-analyses were hand-searched for additional articles. The present search was developed according to the "PRISMA Statement" guidelines (see the PRISMA checklist) (www.prisma-statement.org). For this review, a protocol was not prepared or registered. The search strategy is detailed as follows: (1) Colorectal neoplasms/exp or [(colorectal* or rect* or anal* or anus or colon* or sigmoid) adj3 (cancer* or carcinoma or tumour* or tumor* or neoplas* or adenoma or adenocarcinoma)], abstract (ab), keyword hearing word (kf), original title (ot), title (ti), text word (tw); (2) (observational or case-control or cohort), ab, kf, ot, ti, tw; (3) (incidence or prevalence or risk or odds ratio or hazard ratio), ab, kf, ot, ti, tw; (4) (one-carbon metabolism-related nutrient* or dietary methyl donor* or alcohol), ab, kf, ot, ti, tw; and (5) [gen* or single-nucleotide polymorphism (SNP)* or polymorphism* or methyl metabolizing enzyme* or diet–gene interaction* or hypermethylation* or microsatellite instability*], ab, kf, ot, ti, tw.

Review process and selection criteria

Two researchers independently screened the titles and abstracts of the articles to identify potentially relevant studies. Studies that passed the title/abstract review were retrieved for full-text review. The inclusion and exclusion criteria and the quality of the study were assessed by two researchers with the use of a data extraction form especially designed for this study.

The inclusion criteria consisted of studies: (1) With an observational design (case-control or cohort); (2) that evaluated the exposure to at least one of the following dietary components (dietary nutrient intake and/or plasma levels): Folate, other B vitamins, Met, choline, betaine, and/or alcohol; (3) that included genotyping analyses of methyl-metabolizing enzymes (*MTHFR, MTR,* Met synthase reductase), DN methyltransferase 3 b, euchromatin histone methyltransferase 1 and 2, PR domain zinc finger protein 2; (4) CIMP defined by promotor hypermethylation (calcium voltage-gated channel subunit a1 G, insulin-like growth factor 2, neurogenin1, runt-related transcription factor 3, suppressor of cytokine signalling 1), human *MutL* homolog 1 (*hMLH1*) hypermethylation; (5) MSI using markers [such as mononucleotide microsatellites with quasi-monomorphic allele length distribution in healthy controls but unstable (Bat-26 and/or Bat-25), NR-21, NR-22, NR-24)]; (6) in which the outcome of interest was CRC, colon, or rectal cancer (studies investigating benign adenomas or polyps were excluded); (7) that provided estimates of the adjusted odds ratios or relative risks or hazard ratios with 95% confidence intervals (95%CI); (8) conducted in humans (≥ 18 years old); and (9) written in English or Spanish.

The following types of publications were excluded: (1) Nonoriginal papers (reviews, commentaries, editorials, or letters); (2) meta-analysis studies; (3) off-topic studies; (4) studies lacking specific CRC data; (5) nonhuman research; (6) studies conducted in children, adolescents, or pregnant women; (7) duplicate publications; and (8) low-quality studies (Newcastle-Ottawa scale (NOS)[22] < 4 indicating insufficient study quality).

Study quality assessment

To evaluate the validity of the individual studies, two reviewers worked independently to determine the quality of the included studies based on the use of the NOS for case-control or cohort studies[22]. The maximum score was 9, and a high score (≥ 6) indicated high methodological quality; however, given the lack of studies on the subject under study, it was agreed to select those that had a score equal to or greater than 4. A consensus was reached between the reviewers if there were any discrepancies.

Data extraction

The data extracted for each individual study included the following: Name of the first author, study design, characteristics of the study population (age range or mean age, sex, country), dietary exposure, dietary assessment instrument used, outcomes (including cancer site), comparison, odds ratio or relative risk or hazard ratio (95%CI), adjusted variables, and NOS. For case-control studies, the following additional information was extracted: Number of cases and number of controls. For cohort studies, the following additional information was extracted: Number of participants at baseline, number of CRC cases, and length of follow-up. These variables were judged to be most relevant to the outcome studied. Where multiple estimates for the association of the same outcome were used, the one with the highest number of adjusted variables was extracted. Template data collection forms and data extracted from the included studies will be made available upon request from the corresponding author.

RESULTS

Figure 1 shows the PRISMA flow diagram summarizing the identification and selection of the relevant publications. A total of nineteen studies were included in this systematic review: Fourteen case-control studies[23-36] and five cohort studies[9,37-40]. In total, the case-control studies included 7055 cases and 9032 controls. The cohort studies included 256914 participants, with 1109 cases recorded during follow-up periods that ranged from 7.3 to 22 years. Amongst the case-control studies, nine articles were conducted in the United States, two in the United Kingdom, two in Korea and one in Portugal. With regard to the cohort studies, three were conducted in the Netherlands and two in the United States.

Tables 1 and 2 summarize the main characteristics and findings of the case-control and cohort studies, respectively, on the interactive effect between single-nucleotide polymorphisms in genes encoding methyl-metabolizing enzymes, dietary methyl donors and dietary components that potentially modulate the bioavailability of methyl groups on CRC risk. Tables 3 and 4 show the effects of dietary methyl donors and dietary components that potentially modulate the bioavailability of methyl groups on CRC risk, according to SNPs in genes encoding methyl-metabolizing enzymes and/or mutations in oncogenes, CIMP and/or MSI, in the case-control studies and cohort studies, respectively. Table 5 provides a summary of the results of the studies included in the systematic review.

DISCUSSION

This review summarizes previous studies that have investigated the influence of methyl donor nutrients as epigenetic regulators in CRC. The dietary components that showed a higher association with CRC risk were folate and alcohol. Thus, high folate intake was considered a protective factor, while high alcohol consumption proved to be a risk factor. Several studies have investigated the association between methyl donor nutrients and/or methyl antagonists (*e.g.*, alcohol) and *MTHFR* polymorphisms and have reported significant interactions[23,24,26,29,36]. In one of those case-control studies, those with the *MTHFR* 677 TT genotype, who consume low folate diets, had a greater chance of developing CRC than people with the CC or CT genotype[24]. Two other case-control studies reported that *MTHFR* 677 TT carriers with high (above mean) or adequate folate intake had a low risk of CRC[26,29].

Two case-control studies found that alcohol consumption increased CRC risk among *MTHFR* 677 TT carriers[23,26]. The decreased *MTHFR* enzyme activity among those who carried the T allele, and consumed low methyl donor nutrients and large amounts of alcohol can be utilised to explain the increased CRC risk[25,30]. These dietary habits may alter folate metabolism, especially in people with folate deficiencies[41]. In contrast, several studies have not found an association between *MTHFR* polymorphisms and either folate intake or alcohol intake and CRC risk[25,27]. It has been hypothesized that the differences in folate status among various populations may have influenced the contradictory results on the contribution of *MTHFR* genetic variants in CRC[25]. In addition to *MTHFR* poly-

Table 1 Characteristics of the eight case-control studies included in this systematic review examining the interactive effects between single-nucleotide polymorphisms in genes encoding methyl-metabolizing enzymes and one-carbon metabolism-related dietary compounds on colorectal cancer risk

Ref.	Country	Age (yr)	No. cases (M/W), endpoint	No. controls, type	Gene (SNP)	Nutrient/alcohol	Method for measuring nutrition intake	Outcome (OR, 95%CI)			Adjustments to OR	NOS
								SNP	Nutrient/alcohol	Interaction		
Chen *et al* [23]	United States	40-75	144 M, CRC	627 C	*MTHFR* (677C>T)	Dietary folate, Met, and alcohol	Validated FFQ (self-reported)	No assoc	Alcohol (≥ 5 *vs* ≤ 1 drinks/wk): 1.61 (1.01-2.58)	677TT (*vs* CC/CT)-low alcohol consumption (≤ 1 drinks/wk): 0.11 (0.01-0.85) (*P-interac* = 0.02)	Age, CRC family history	7
Guerreiro *et al*[24]	Portugal	Cases (64.2 ± 11.3), controls (62.2 ± 12.1)	104/92 CRC	200 C	*MTHFR* (677C>T), *MS* (2756A>G), *SHMT* (1420C>T)	Dietary folate, vitamins B_6 and B_{12}, glycine, Met, serine, and alcohol	Validated FFQ (by interview)	677TT (*vs* CC/CT): 3.01, (1.3-6.7); 1420TT (*vs* CC/CT): 2.6, (1.1-5.9)	Folate (> 406.7 mcg/d *vs* < 406.7 mcg/d): 0.67 (0.45-0.99)	677TT (*vs* CC/CT)– folate (< 406.7 mcg/d): 14.0, 1.8-108.5 (*P* = 0.05)	Age, CRC history, and sex	5
Kim *et al* [25]	Korea	30-79	465/322 CRC (363 CCa, 330 RCa)	656 H	*MTHFR* (677C>T)	Dietary folate and alcohol	Validated FFQ	677TT (*vs* CC/CT): 0.60 (0.46-0.78)	Folate (high *vs* low intake): 0.64 (0.49-0.84); alcohol (high *vs* low intake): 1.76 (1.26-2.46)	677CC/CT–Low-methyl diet (folate < 209.69 mcg/d and alcohol ≥ 30 g/d): 2.32 (1.18-4.56) (*P-interac* = no assoc)	Age, BMI, CRC family history, energy intake, multivitamin use, sex, smoking status	7
Ma *et al* [26]	United States	40-84	202 M, CRC	326 C	*MTHFR* (677C>T)	Plasma folate, and alcohol consumption	FFQ (self-reported)	677TT (*vs* CC): 0.45 (0.24-0.86)	Folate (plasma deficiency *vs* adequate levels): No assoc	677TT (*vs* TC/CC)–folate (adequate levels): 0.32 (0.15-0.68)	Age, alcohol consumption, aspirin use, BMI, exercise, multivitamin use, and smoking status	7
									Alcohol: Unk	677TT (*vs* CC)–alcohol (0-0.14 drinks/d): 0.12 (0.03-0.57)	Age	
Murtaugh *et al*[27]	United States	30-79	446/305 RCa	979 C	*MTHFR* (677C>T, 1298A>C)	Dietary folate, riboflavin, vitamins B_6 and B_{12}, Met, and alcohol	FFQ (by interview)	W, 677TT (*vs* CC): 0.54 (0.30-0.98). M&W, 1298CC (*vs* AA): 0.67 (0.46-0.98)	Dietary folate (> 475 mcg/d *vs* < = 322 mcg/d): 0.66 (0.48-0.92). High methyl donor status (*vs* low status): 0.79 (0.66-0.95)	No assoc	Age, BMI, ibuprofen use, intake of energy, fibre and calcium, PA, sex, and smoking status	6
Pufulete *et al*[28]	United Kingdom	38-90	13/15 CRC	76 C	*MTHFR* (677C>T, 1298A>C), *MS* (2756A>G), *CBS* (844ins68)	Plasma folate, vitamin B_{12}, and homocysteine, alcohol and folate intake	Validated FFQ (by interview)	677TT (*vs* CC): 5.98 (0.92-38.66), *P* = 0.06; 1298CC (*vs* AA): 12.6 (1.12-143.70), *P* = 0.04	Folate status score (T_3 *vs* T_1): 0.09 (0.01-0.57), *P-trend* = 0.01	Unk	Age, alcohol consumption, BMI, sex, and smoking status	7
Sharp *et al* [29]	United Kingdom		150/114 (189 CCa, 75 RCa)	408C	*MTHFR* (677C>T,	Dietary folate, riboflavin, vitamins B_6	Validated FFQ (self-reported)	No assoc	No assoc	677CT/TT (*vs* CC)–folate (> mean): *P-interac* =	Age, CRC family history, energy	6

					1298A>C)	and B_{12}, and alcohol				0.029. 677CT/TT (*vs* CC)–vitamin B_6 (> mean): *P-interac* = 0.016	intake, NSAID use, PA, and sex	
Slattery *et al*[30]	United States	30-79	824/849 CC (DCCa 405/303; PCCa 395/327)	1816 C	*MTHFR* (677C>T)	Dietary folate, Met, vitamins B_6 and B_{12}, and alcohol	Validated CARDIA diet questionnaire	677TT: No assoc	Unk	TT-low risk diet (high in folate and Met and without alcohol): 0.4 (0.1-0.9)	Age, BMI, intake of energy and fibre, PA, and smoking intensity	7

Asso: Significant association; BMI: Body mass index; C: Community controls; CBS: Cystathionine synthase; CCa: Colon cancer; CI: Confidence interval; CRC: Colorectal cancer; DCCa: Distal colon cancer; FFQ: Food frequency questionnaire; H: Hospital controls; interac: Interaction; M: Men; Met: Methionine; *MS*: Methionine synthase; *MTHFR*: Methylenetetrahydrofolate reductase; NSAID: Nonsteroidal anti-inflammatory drugs; NOS: Quality Newcastle-Ottawa Scale; OR: Odds ratio; PA: Physical activity; PCCa: Proximal colon cancer; RCa: Rectal cancer; *SHMT*: Serine hydroxymethyltransferase; SNP: Single-nucleotide polymorphism; unk: Unknown; W: Women.

Table 2 Characteristics of the cohort study included in this systematic review examining the interactive effects between single-nucleotide polymorphisms in genes encoding methyl-metabolizing enzymes and one-carbon metabolism-related dietary compounds on colorectal cancer risk

Ref.	Country	Study cohort (age, yr)	No. participants (M/W)	No. incident cases	Follow-up length, y	Gene (SNP)	Nutrient/alcohol	Method for measuring nutrition intake	Outcome (RR, 95%CI)			Adjustments to RR	NOS
									SNP	Nutrient/alcohol	Interaction		
de Vogel *et al*[9]	Netherlands	Netherlands Cohort Study on diet and cancer (55-69)	58279/62573	734 CRC	7.3	*MTHFR* (rs1801133, rs1801131), *MTR* (rs1805087), *MTRR* (rs1801394), *DNMT3B* (rs2424913, rs406193), *EHMT1* (rs4634736), *EHMT2* (rs535586), *PRDM2* (rs2235515)	Dietary folate, Met, vitamins B_2 and B_6, alcohol	Validated FFQ (self-reported)	Unk	Unk	≤ 1 rare allele in folate metabolizing enzymes–vitamin B_2 (T_3 *vs* T_1): 0.30, (0.11-0.81), *P-trend* = 0.005. Rare allele of *DNMT3B* C>T (rs406193)–vitamin B_6 (T_3 *vs* T_1): 1.90 (1.00-3.60), *P-trend* = 0.04. Common allele of *PRDM2* G>A (rs2235515)–vitamin B_6 (T_3 *vs* T_1): 1.49 (1.00-2.22), *P-trend* = 0.03. No assoc	Age, alcohol consumption, BMI, CRC family history, intake of energy and alcohol, sex, and smoking status	9

Asso: Significant association; BMI: Body mass index; CI: Confidence interval; CRC: Colorectal cancer; *DNMT3B*: ADN (cytosine-5-)-methyltransferase 3 ; *EHMT1*: Euchromatin histone methyltransferase 1; FFQ: Food frequency questionnaire; M: Men; *MTHFR*: Methylenetetrahydrofolate reductase; *MTR*: Methionine synthase; *MTRR*: Methionine synthase reductase; NOS: Quality Newcastle-Ottawa Scale; *PRDM2*: PR domain zinc finger protein 2; RR: Relative risk; SNP: Single-nucleotide polymorphism; T: Tertile; unk: Unknown; W: Women.

morphisms, de Vogel *et al*[9] also investigated the modifying effects of polymorphisms of Met synthase reductase, *MTR*, DN methyltransferase 3 b, euchromatin histone methyltransferase 1 and 2, and PR domain zinc finger protein 2 but found no interactions, although some *P-trends* were significant.

Regarding the methylation abnormalities of genes, de Vogel *et al*[37] found that high vitamin B_6 intake was associated with an increased CRC risk caused by hypermethylation of the *hMLH1* promoter among men. Therefore, these authors suggest that vitamin B_6 may have had a tumour-promoting effect by increasing promoter methylation. However, the intake of folate, vitamin B_2, Met and alcohol was not associated with the risk of tumours showing *hMLH1* hypermethylation. In any case, other studies

Table 3 Characteristics of the six case-control studies included in this systematic review examining the interactive effects between single-nucleotide polymorphisms in genes encoding methyl-metabolizing enzymes and/or mutations in oncogenes, CpG island methylator phenotype and/or microsatellite instability, and one-carbon metabolism-related dietary compounds on colorectal cancer risk

Ref.	Country	Age (yr)	No. cases (M/W), endpoint	No. controls and type	Gene (SNP)	CIMP markers	MSI	Nutrient/alcohol	Method for measuring nutrition intake	Outcome (OR, 95%CI), interaction		Adjustments to OR	NOS
										CIMP markers/MSI–nutrient/alcohol	CIMP markers-/MSI–SNP–nutrient/alcohol		
Busch *et al*[31]	United States	40-80 (AAs *vs* EAs)	244/241 CRC	Analyses were only performed in tumour tissue		*CACNA1G, hMLH1, NEUROG1, RUNX3, SOCS1*	Unk	Dietary folate and alcohol	Unk	EAs: High *CACNA1G* methylation tumour (cut point of 5%)–high folate intake: 0.3 (0.14-0.66); high *SOCS1* methylation tumour (cut point of 3%)–high folate intake: 0.3 (0.11-0.80)	Unk	-	4
Curtin *et al*[32]	United States	30-79	518/398 CCa	1972 C	*MTHFR* (677C>T, 1298A>C), *TS* variants (*TSER, TTAAAG* in 3´-UTRs 1494), *MTR* (919D>G), *RFC* (80G>A), *MTHFD1* (R134K, R653Q), *ADH3* (1045A>G)	*MINT1, MINT2, MINT31, p16, hMLH1*	Unk	Dietary folate, Met, vitamin B_{12}, and alcohol	Adaptation of the CARDIA diet history	Unk	*MTHFR* 1298AA–alcohol (high *vs* none): CIMP+, 0.5 (0.3-0.97), $P < 0.01$; *ADH3* (1 or 2 variant, slow catabolizing*2 *vs* homozygous for the common allele)–folate (low): CIMP+, 1.6 (1.03-2.6), $P = 0.02$. *MTHFR* 1298AC or CC-high-risk dietary pattern (low in folate or Met intake, high in alcohol): CIMP+, 2.1 (1.3-3.4), $P = 0.03$	Age, centre, other SNPs, sex, smoking intensity, and race	9
Curtin *et al*[33]	United States	30-79	559/392	1205 C	*MTHFR (* 1298A>C), *TP53, KRAS2,*	*CDKN2A, hMLH1, MINT 1, 2* and *31*		Folate, riboflavin, vitamins B_6, B_{12}, and Met	Adaptation of the CARDIA diet history (by interview)	M: Folate (T_3 *vs* T_1)–CIMP+, 3.2 (1.5-6.7), $P < 0.01$	1298 AC/CC (*vs* AA)–folate (T_3 *vs* T_1): 0.4 (0.2-1.0), $P = 0.04$, for CIMP+	Age, centre, intake of energy and fibre, NSAID use, oestrogen use (W), PA, race, referent year, sex, screening, and smoking	8
Kim *et al*[34]	Korea	30-79	465/322 CRC (363 CCa, 330 RCa)	656 H	*MTHFR* (677C>T)	Unk	2 mononuc-leotide markers (Bat25 and Bat26) and 3 dinucleotide markers (D2S123, D5S346, and D17S250)	Folate, vitamins B_2, B_6, B_{12}, niacin, Met, and choline	Validated FFQ	Unk	DCCa: *hMSH3* (rs41097) AG/GG (*vs* AA)–niacin (> 14.00 mg/d *vs* < 14.00 mg/d)–MSI–MMR status: 0.49 (0.28-0.84), *P-interac* = 0.008	Age, intake of energy and alcohol, BMI, CRC family history, educational level, occupation, income, PA,	7

											sex, and smoking status	
Slattery *et al*[35]	United States	30-79	821/689 CRC	2410 C	Unk	Unk	10 tetranucleotide repeats, 3 Bat-26 and TGFbRII	Dietary folate, and alcohol	Validated CARDIA diet questionnaire	Alcohol–MSI+ (*vs* MSI-): 1.6 (1.0-2.5), *P-trend* = 0.03; liquor–MSI+ (*vs* MSI-): 1.6 (1.1-2.4), *P-trend* = 0.02		Age, BMI, intake of energy, fibre and calcium, intake, PA, sex	7
Slattery *et al*[36]	United States	30-79	638/516 CRC	2410 C	*BRAF* (V600E)	*MINT1*, *MINT2*, *MINT31*, *p16* and *hMLH1*	Unk	Folate, vitamins B_6 and B_{12}, Met, and alcohol	Diet history questionnaire	No assoc	MSI tumour–alcohol (high *vs* none): 1.6 (0.9-2.9), *P-trend* = 0.04, for *p16* unmethylated; 1.7 (0.7-4.3), *P-trend* = 0.06, for $CIMP_{low}$ (< 2 markers); 2.2 (1.2-3.7), *P-trend* = 0.01, for *BRAF* wildtype	Age, alcohol intake, BMI, intake of energy and folate, density of calcium and fibre, NSAIDs use, PA, sex, smoking intensity	7

AA: African Americans; *ADH3*: Alcohol dehydrogenase 3; asso: Significant association; Bat: Mononucleotide microsatellite with quasi-monomorphic allele length distribution in healthy controls but unstable; BMI: Body mass index; *BRAF*: B-Raf proto-oncogene; C: community controls; *CACNA1G*: Calcium voltage-gated channel subunit a1 G; CCa: Colon cancer; *CDKN2A*: Cyclin-dependent kinase inhibitor 2A; CI: Confidence interval; CIMP: CpG island methylator phenotype; CRC: Colorectal cancer; EA: European Americans; FFQ: Food frequency questionnaire; H: Hospital controls; *hMLH1*: Human *MutL* homolog 1; *hMSH3*: Human *MutS* homolog 3; interact: Interaction; *KRAS:* Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; M: Men; Met: Methionine; MINT: Methylated in tumours; MMR: Mismatch repair; MSI: Microsatellite instability; *MTHFD:* Metilentetrahidrofolate dehydrogenase; *MTHFR:* Methylenetetrahydrofolate reductase; *MTR:* Methionine synthase; *NEUROG1*: Neurogenin1; NOS: Quality Newcastle-Ottawa Scale; NSAID: Nonsteroidal anti-inflammatory drugs; OR: Odds ratio; PA: Physical activity; RCa: Rectal cancer; *RFC*: Reduced folate carrier; *RUNX3:* Runt-related transcription factor 3; SNP: Single-nucleotide polymorphism; *SOCS1*: Suppressor of cytokine signalling 1; T: Tertile; *TGFbRII*: Transforming growth factor β receptor type II; TS: Thymidylate synthase; *TSER:* Thymidylate synthase enhancer region; TP53: Tumour protein p53; unk: Unknown; UTR: Untranslated region; W: Women.

showed inverse associations between vitamin B_6 intake and CRC risk, both when the intake level was higher[6] and when it was similar[42] to that in the study of de Vogel *et al*[37]. Therefore, further attention should be given to this association in future studies.

Another interesting finding in this review is the inverse association between dietary folate and Met and B-Raf proto-oncogene mutations among men[37]. A previous study showed that folate may increase the risk of tumours harbouring truncating adenomatous polyposis coli mutations in men[43]. Apparently, relatively high folate intake may confer a growth advantage to mutated tumours independent of the type of mutation. Nevertheless, the occurrence of MSI does not seem to be sensitive to methyl donor intake or to that of alcohol[36-37,44].

In one of the case-control studies that we analysed, an interaction was observed between a high- or low-risk diet and *MTHFR* 1298A>C (but not 677C>T) with regard to CIMP status[32]. This result suggests that a genetic polymorphism at *MTHFR* 1298A>C interacts with the diet (with a low folate and Met intake and a high alcohol consumption) increasing the risk of highly CpG-methylated colon tumours. S-adenosylmethionine binds as an allosteric inhibitor in the *MTHFR* regulatory region, which is where the 1298A>C variant is found. This gives some justification for the stronger associations between *MTHFR* 1298A>C and CIMP rather than the 677C>T SNP, which has an opposite effect on the stability of the enzyme. Due to their high linkage disequilibrium, the *MTHFR* 677C>T and 1298A>C polymorphisms should not be viewed separately. van Engeland *et al*[40] also discovered that people

Table 4 Characteristics of the five cohort studies included in this systematic review examining the interactive effects between single-nucleotide polymorphisms in genes encoding methyl-metabolizing enzymes and/or mutations in oncogenes, CpG island methylator phenotype and/or microsatellite instability, and one-carbon metabolism-related dietary compounds on colorectal cancer risk

Ref.	Country	Study cohort (age, yr)	No. participants (M/W)	No. incident cases	Follow-up length, yr	Gene (SNP)	CIMP markers	MSI	Nutrient/alcohol	Method for measuring nutrition intake	Outcome (RR, 95%CI) interaction			NOS
											CIMP markers/MSI–nutrient/alcohol	CIMP markers–/MSI–SNP–nutrient/alcohol	Adjustments to RR	
de Vogel *et al* [9]	Netherlands	The Netherlands Cohort Study on diet and cancer (55-69)	58279/62573	734 CRC	7.3	*MTHFR* (rs1801133, rs1801131), *MTR* (rs1805087), *MTRR* (rs1801394), *DNMT3B* (rs2424913, rs406193), *EHMT1* (rs4634736), *EHMT2* (rs535586), *PRDM2* (rs2235515)	*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, *SOCS1*	Bat-26, Bat-25, NR-21, NR-22, NR-24	Dietary folate, Met, vitamins B_2 and B_6, alcohol	Validated FFQ (self-reported)	No assoc	Unk	BMI, CRC family history, intake of energy and alcohol, sex, and smoking status	9
de Vogel *et al* [37]	Netherlands	The Netherlands Cohort Study on diet and cancer (55-69)	58279/62573	734 CRC	7.3	*BRAF* (V600E)			Dietary folate, Met, vitamins B_2 and B_6, alcohol	Validated FFQ (self-reported)	M: *BRAF* mut–folate (T_3 *vs* T_1): 3.04 (1.13-8.20), *P-trend* = 0.03; *BRAF* mut–Met (T_3 *vs* T_1): 0.28 (0.09-0.86), *P-trend* = 0.02); *hMLH1* hypermethylation–vitamin B_6 (T_3 *vs* T_1): 3.23 (1.15-9.06), *P- trend* = 0.03	Unk	Age, BMI, CRC family history, intake of energy, meat, total fat, fibre, vitamin C, total iron and calcium, smoking status	9
Schernhammer *et al*[38]	United States	The Nurses' Health Study (W) (30-55) and the Health Professional Follow-up Study (M) (40–75)	47371/88691	669 CCa	22	*KRAS*	Unk	D2S123, D5S346, D17S250, Bat25, Bat26 (14), Bat40, D18S55, D18S56, D18S67, D18S487	Folate, vitamins B_6 and B_{12}, Met, and alcohol	Validated FFQ (self-reported)	Unk	MSI/*KRAS*–folate: No assoc for CCa. MSI/*KRAS*–vitamins B_6 or B_{12}: No assoc for CCa	Age, aspirin use, smoking, BMI, colon polyps, CRC family history, intake of alcohol, energy, beef, calcium, vitamins B_6 and B_{12}, and Met, multivitamin use, PA, sex, screening sigmoidoscopy	9

Schernhammer *et al*[39]	United States	The Nurses' Health Study (30-55)	88691 W	375 CCa		*BRAF*	*CHFR, MGMT, p14, WRN, HTC1, MINT1, MINT31, IGFBP3*	Unk			Folate (Q_4 *vs* Q_1: No assoc with CIMP–high tumour risk; and no assoc with *BRAF* status	Unk			
van Engeland *et al*[40]	Netherlands	Netherlands Cohort Study on Diet and Cancer (55-69)	58279/62573	122 CRC	7.3	Unk	*APC-1A, p14ARF, p16INK4A, hMLH1, O 6-MGMT,* and *RASSF1A*	Unk	Dietary folate and alcohol	Validated FFQ (self-reported)	Low *vs* high-methyl donor intake–promoter methylation (> 1 gene methylated): No assoc	Unk		Age, CRC family history, intake of energy, fibre, vitamin C, and iron, sex	9

APC: Adenomatous polyposis coli; asso: Significant association; Bat: Mononucleotide microsatellite with quasi-monomorphic allele length distribution in healthy controls but unstable; BMI: Body mass index; *BRAF*: B-Raf proto-oncogene; *CACNA1G:* Calcium voltage-gated channel subunit a1 G; CCa: Colon cancer; *CHFR*: RING finger domain protein; CI: Confidence interval; CIMP: CpG island methylator phenotype; CRC: Colorectal cancer; *DNMT3B:* DNA methyltransferase 3 *EHMT:* Euchromatin histone methyltransferase; FFQ: Food frequency questionnaire; *hMLH1*: Human *MutL* homolog 1; *HTC*: Histidine triad with channel; IGF2: Insulin-like growth factor 2; *IGFBP*: Insulin-like growth factor-binding protein; *KRAS:* Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; M: Men; Met: Methionine; *MGMT*: DNA repair enzyme O(6)-methylguanine-DNA methyltransferase; MINT: Methylated in tumours; MSI: Microsatellite instability; *MTHFR:* Methylenetetrahydrofolate reductase; *MTR:* Methionine synthase; *MTRR*: Methionine synthase reductase; mut: Mutation; *NEUROG1*: Neurogenin1; NOS: Quality Newcastle-Ottawa Scale; PA: Physical activity; *PRDM2*: PR domain zinc finger protein 2; Q: Quartile; RASSF1A: Ras association domain family 1 isoform A; RR: Relative risk; *RUNX3:* Runt-related transcription factor 3; SNP: Single-nucleotide polymorphism; *SOCS1*: Suppressor of cytokine signalling 1; T: Tertile; unk: Unknown; W: Women; *WRN*: Werner syndrome gene.

diagnosed with CRC, with low intake of folate and high consumption of alcohol, had a greater prevalence of promoter hypermethylation; however, the difference was not statistically significant due to limited power. Therefore, it was proposed that stratification for functionally significant SNPs in the genes encoding folate metabolism enzymes could strengthen the observed effect of folate deficiency on promoter methylation[40].

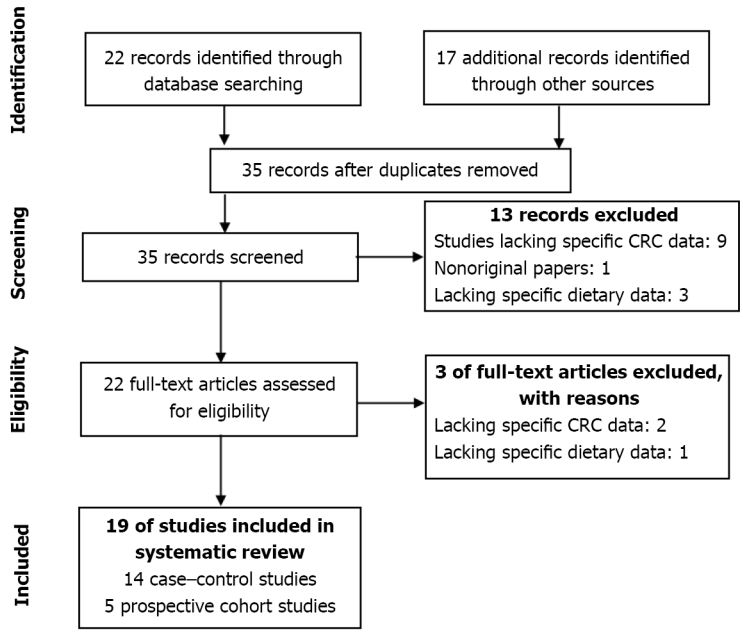
Additionally, Curtin *et al*´s study[32] found an interaction between alcohol consumption and *MTHFR* 1298A>C in association with CIMP status. Relative to the AA genotype in non-drinkers, the *MTHFR* 1298 AA genotype was linked to a higher risk of CIMP+ in drinkers. In a previous study by Slattery *et al* [35], in which associations between CIMP status and alcohol use were assessed without taking into regard to genotype, no association was observed. These results imply that the activity of the 1C-metabolism enzyme may alter the risk associated with alcohol in determining the CIMP status of colon cancer. In any case, to date, few published studies have evaluated, CIMP in CRC for possible relationships with 1C-metabolism SNPs[32,45,46]. Moreover, these data raise the possibility that more investigation is required to clarify the function of genetic SNPs in relation to CIMP status and the promoter hypermethylation of particular genes.

In one case-control study[36], an association between long-term alcohol consumption and increased likelihood of having a CIMP-low or B-Raf proto-oncogene-mutated tumour was observed. Among those with unstable tumours, they observed that alcohol was more likely to be associated with CIMP-low rather than CIMP-high tumours. A previous study of these same authors showed that alcohol increased the risk for MSI+ tumours in general[35]. Therefore, these findings suggest that the increased risk of MSI associated with alcohol is limited to those tumours that are unmethylated rather than methylated.

Table 5 Summary of results of the studies included in this systematic review

Gen, SNP/CIMP/MSI	Nutrients/alcohol	CRC risk/CIMP+	Ref.
MTHFR 677 TT	Folate; Adequate folate	CRC risk; CRC risk	Guerreiro *et al*[24]; Sharp *et al*[29]; Ma *et al*[26]
	Alcohol	CRC risk	Chen *et al*[23]; Ma *et al*[26]
	Folate/Met, and without alcohol	CRC risk	Slattery *et al*[30]
	Vitamin B_6	CRC risk	Sharp *et al*[29]
BRAF mutation	Folate	CRC risk (M)	de Vogel *et al*[37]
	Met	CRC risk (M)	
hMLH1 hypermethylation	Vitamin B_6	CRC risk (M)	
MTHFR 1298 AC/CC	Folate/Met, and alcohol	CIMP+	Curtin *et al*[32]
MTHFR 1298 AA	Alcohol	CIMP+ (CCa)	
p16 unmethylated, $CIMP_{low}$ or *BRAF* mut	Alcohol	CRC risk	Slattery *et al*[36]
hMSH3, MSI or MMR status	Niacin	CRC risk (DCCa)	Kim *et al*[34]

BRAF: B-Raf proto-oncogene; CCa: Colon cancer; CRC: Colorectal cancer; CIMP: CpG island methylator phenotype; DCCa: Distal colon cancer; h*MLH1*: Human *MutL* homolog 1; *hMSH3:* Human *MutS* homolog 3; M: Men; Met: Methionine; MMR: Mismatch repair; MSI: Microsatellite instability; *MTHFR:* Methylenetetrahydrofolate reductase; mut: Mutation; SNP: Single-nucleotide polymorphism.
↑: High intake or increased risk or high probability.
↓: Low intake or increased risk.



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Figure 1 PRISMA flow diagram summarizing the identification and selection of the relevant publications assessing the influence of methyl donor nutrients as epigenetic regulators in colorectal cancer. CRC: Colorectal cancer.

Finally, regarding the interaction between MMR SNPs and methyl donor nutrient intake on CRC based on MSI status, in a case-control study[34], a strong inverse association was observed for *hMSH3* AG or GG carriers with a high intake of niacin, particularly among patients with CC and microsatellite stability or proficient MMR status. However, to ascertain the processes behind this association with CRC risk, the precise roles of this SNP must be identified.

The importance of interactions between modifiable factors, such as methyl donor nutrients and CRC, is suggested by evidence that colorectal carcinogenesis is generated by numerous molecular pathways in parallel with MSI status, which is caused by a defect in the MMR machinery. In this sense, it is worth

remembering the article mentioned by de Vogel *et al*[37], in which it was reported that high consumption of vitamin B_6 was linked to an increased risk of sporadic CRC with *hMLH1* hypermethylation, indicating that vitamin B_6 affects CRC risk through both genetic and epigenetic mechanisms. Based on the findings of Kim *et al*[34], it may be possible to explain the interactions between dietary methyl donor nutrients, such as niacin, and *hMSH3* genetic variants as predictors of CRC risk.

DNA methylation and microRNA expression levels may be regulated by the MMR machinery's epigenetic relationships with methyl groups[47]. Through the methylation of CpG islands in the promoter region, 1C metabolism mediated by methyl donor nutrients can change how the DNA MMR system is activated. A adequate DNA MMR system with methyl groups may control the equilibrium between the repair and accumulation of short repeat sequences, preventing extensive DNA damage that supports colorectal carcinogenesis.

This systematic review has several strengths: (1) Cohort and case-control studies were identified through a systematic search; (2) a quantitative NOS scale was used to assess the quality of the studies; and (3) most studies (twelve of nineteen) used a validated questionnaire to assess dietary intake. To our knowledge, this is the first systematic review regarding the influence of methyl donor nutrients as epigenetic regulators in CRC.

The limitations of this review include the following: (1) Case-control studies, which are susceptible to recall and selection bias, made up the bulk of the research. However, most studies were based on community controls; thus, they might be a good representation of the frequency of genetic variants or of dietary habits of the overall population; (2) the heterogeneous nature of studies, including the study population characteristics, sample size, study design, and follow-up periods; (3) potential residual confounding because of the observational nature of the studies included or the possibility that not all the studies were adjusted for important nutrient variables; (4) some of the dietary assessments were self-reported, which may affect the reliability of the reported intakes, although the use of validated questionnaires in most studies could reduce this bias; (5) some studies had a relatively limited sample size or effect size, which made it difficult for them to detect the interactions between genetic and nutritional information, which could explain, in part, the lack of results with a statistically significant level; and (6) despite the fact that some research have found differential interactions based on the CRC subtype[34], the majority of studies lacked stratified analysis. Considering these limitations, the conclusions from these researchers should be taken carefully. Consequently, it is difficult for this review to explain the gene-diet interactions and their effects on the development of CRC.

CONCLUSION

In this systematic review of observational studies, some interactions between *MTHFR* polymorphisms, methyl donor nutrients (such as folate) and alcohol on CRC risk are suggested. Moreover, some studies show that vitamin B_6, niacin and alcohol may affect CRC risk through not only genetic but also epigenetic regulation. In any case, this review was not able to clarify which mechanisms underlie the influence of methyl donor nutrients on DNA methylation, as well as the efficacy of methyl uptake, transportation, and the final involvement in methyl-related gene expression. Further prospective studies with large samples and long follow-up periods, as well as clinical trials that take into account the long latency period of CRC, are needed to clarify the influence of methyl group donors as epigenetic regulators, with particular emphasis on differences in CRC subsite-specific risk. Such studies may provide valuable insight into the biological mechanisms with the goal of identifying at-risk subpopulations and promoting primary prevention of CRC.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer (CRC) is the third most frequent cancer and is responsible for the second-highest mortality rate in cancer patients worldwide. The main factors which may help prevent CRC are those associated with diet, lifestyle, and prevention of metabolic diseases. With regards to the dietary component, one-carbon metabolism-related nutrients have been considered anticarcinogenic and chemotherapeutic agents in the one-carbon metabolic network. However, it is still unclear whether the influence of methyl donor intake is modified by polymorphisms in these epigenetic regulators.

Research motivation

Although screening for early detection of CRC is effective to help decrease the trends in mortality rates, understanding daily life factors is also important to prevent this type of cancer. A better understanding of the molecular basis of CRC could contribute to a better design of future research and better preventive nutritional management in this type of cancer.

Research objectives

In the present work, we reviewed previous studies that have investigated this matter to improve the current understanding of the molecular basis of CRC.

Research methods

A literature search in the Medline database, *Reference Citation Analysis* (https://www.referencecitation-analysis.com/, an artificial intelligence technology-based open multidisciplinary citation analysis database), and manual reference screening were performed to identify observational studies published from inception to May 2022. A search for relevant keywords and medical subject heading terms related to dietary methyl donors, dietary components that potentially modulate the bioavailability of methyl groups, genetic variants of methyl-metabolizing enzymes, markers of CpG island methylator phenotype and/or microsatellite instability, in combination with keywords related to CRC events was conducted. The present search was developed according to the "PRISMA Statement" guidelines. To evaluate the validity of the individual studies, two reviewers worked independently to determine the quality of the included studies based on the use of the Newcastle-Ottawa scale for case-control or cohort studies.

Research results

A total of fourteen case-control studies and five cohort studies were identified. In total, the case-control studies included 7055 cases and 9032 controls. The cohort studies included 256914 participants, with 1109 cases recorded during follow-up periods that ranged from 7.3 to 22 years. The dietary components that showed a higher association with CRC risk were folate and alcohol. Thus, high folate intake was considered a protective factor, while high alcohol consumption proved to be a risk factor. Several studies have investigated the association between methyl donor nutrients and/or methyl antagonists (*e.g.*, alcohol) and methylenetetrahydrofolate reductase (*MTHFR*) polymorphisms and have reported significant interactions. In one of those case-control studies, those with the *MTHFR* 677 TT genotype, who consume low folate diets, had a greater chance of developing CRC than people with the CC or CT genotype. Two other case-control studies reported that *MTHFR* 677 TT carriers with high (above mean) or adequate folate intake had a low risk of CRC.

Research conclusions

In this systematic review of observational studies, some interactions between *MTHFR* polymorphisms, methyl donor nutrients (such as folate), and alcohol on CRC risk are suggested. Moreover, some studies show that vitamin B_6, niacin, and alcohol may affect CRC risk through not only genetic but also epigenetic regulation.

Research perspectives

This review was not able to clarify which mechanisms underlie the influence of methyl donor nutrients on DNA methylation, as well as the efficacy of methyl uptake, transportation, and the final involvement in methyl-related gene expression. Further prospective studies with large samples and long follow-up periods, as well as clinical trials that consider the long latency period of CRC, are needed to clarify the influence of methyl group donors as epigenetic regulators, with particular emphasis on differences in CRC subsite-specific risk.

FOOTNOTES

Author contributions: The study was conceived and designed by Chávez-Hidalgo LP, Martín-Fernández-de-Labastida S, M de Pancorbo M, and Arroyo-Izaga M; The data were acquired, collated, and analysed by Chávez-Hidalgo LP and Arroyo-Izaga M; The study was drafted and revised critically for important intellectual content by all authors; The work reported in the paper has been performed by the authors, unless clearly specified in the text; All authors gave final approval of the version to be published and have contributed to the study; No ethical approval was needed.

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Country/Territory of origin: Spain

ORCID number: Lourdes Pilar Chávez-Hidalgo 0000-0002-2766-9009; Silvia Martín-Fdz-de-Labastida 0000-0003-2635-5814; Marian M de Pancorbo 0000-0002-8081-0702; Marta Arroyo-Izaga 0000-0001-5592-4241.

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CASE REPORT

Percutaneous transhepatic intraportal biopsy using gastroscope biopsy forceps for diagnosis of a pancreatic neuroendocrine neoplasm: A case report

Guang-Chuan Wang, Guang-Jun Huang, Chun-Qing Zhang, Qian Ding

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Guang-Chuan Wang, Guang-Jun Huang, Chun-Qing Zhang, Qian Ding, Department of Gastroenterology, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan 250021, Shandong Province, China

Corresponding author: Qian Ding, MD, Chief Physician, Doctor, Department of Gastroenterology, Shandong Provincial Hospital Affiliated to Shandong First Medical University, No. 324 Jingwu Weiqi Road, Jinan 250021, Shandong Province, China.
dq224@163.com

Abstract

BACKGROUND

Pancreatic neuroendocrine neoplasms (PNENs) are a rare group of neoplasms originating from the islets of the Langerhans. Portal vein tumor thrombosis has been reported in 33% of patients with PNENs. While the histopathological diagnosis of PNENs is usually based on percutaneous biopsy or endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA), these approaches may be impeded by gastric varices, poor access windows, or anatomically contiguous critical structures. Obtaining a pathological diagnosis using a gastroscope biopsy forceps *via* percutaneous transhepatic intravascular pathway is an innovative method that has rarely been reported.

CASE SUMMARY

A 72-year-old man was referred to our hospital for abdominal pain and melena. Abdominal contrast-enhanced magnetic resonance imaging revealed a well-enhanced tumor (size: 2.4 cm × 1.2 cm × 1.2 cm) in the pancreatic tail with portal vein invasion. Traditional pathological diagnosis *via* EUS-FNA was not possible because of diffuse gastric varices. We performed a percutaneous transportal biopsy of the portal vein tumor thrombus using a gastroscope biopsy forceps. Histopathologic examination revealed a pancreatic neuroendocrine neoplasm (G2) with somatostatin receptors 2 (+), allowing systemic treatment.

CONCLUSION

Intravascular biopsy using gastroscope biopsy forceps appears to be a safe and effective method for obtaining a histopathological diagnosis. Although well-designed clinic trials are required to obtain more definitive evidence, this procedure may help improve the diagnosis of portal vein thrombosis and related

diseases.

Key Words: Percutaneous transhepatic intravascular biopsy; Portal vein tumor thrombosis; Gastroscope biopsy forceps; Pancreatic neuroendocrine neoplasms; Case report

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Core Tip: Endoscopic ultrasound-guided fine-needle aspiration or percutaneous biopsy of pancreatic neuroendocrine neoplasms patients can be impeded by gastric varices and poor access windows. In this patient, we faced the challenge of obtaining biopsy of the pancreatic mass which had invaded the portal vein. We performed a percutaneous transportal biopsy of the portal vein tumor thrombosis using a gastroscope biopsy forceps. Our experience suggests that gastroscope biopsy forceps is a viable alternative to obtain biopsy in the portal vein system. Our work expands the use of diagnostic transhepatic portal catheterization. This method may also help prevent repeated liver puncture, reducing the risk of complications.

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INTRODUCTION

Pancreatic neuroendocrine neoplasms (PNENs) are rare tumors accounting for approximately 2% of all pancreatic tumors[1]. Portal vein tumor thrombosis (PVTT) was reported in approximately 33% of patients with non-functioning PNENs (NF-PNENs)[2]. Tumor thrombosis in such cases may cause splenoportomesenteric hypertension, potentially resulting in life-threatening upper gastrointestinal bleeding. Computed tomography (CT) or magnetic resonance imaging (MRI) is useful for diagnosis. Tissue samples are usually obtained *via* imaging-guided percutaneous fine-needle aspiration biopsy or core needle biopsy, endoscopic retrograde cholangiopancreatography, or endoscopic ultrasound-guided fine-needle aspiration/biopsy (EUS-FNA/B)[3]. This report describes the first diagnostic case of percutaneous transhepatic intravascular biopsy of PVTT caused by PNEN invasion using a gastroscope biopsy forceps in a patient with severe gastric varices in whom diagnosis could not be made through percutaneous or endoscopic approaches.

CASE PRESENTATION

Chief complaints

A 72-year-old man was referred to a local hospital for upper abdominal pain with melena for 10 d.

History of present illness

He was diagnosed with pancreatitis and gastric varices. The abdominal pain was relieved after somatostatin treatment for 2 d. The patient was referred to our hospital for further treatment.

History of past illness

Six months ago, he was diagnosed with gastric varices after undergoing gastroscopy as part of routine medical checkup; however, he did not undergo further evaluation.

Personal and family history

He had no family history of malignant tumors, psychological, or genetic disorders.

Physical examination

Physical examination showed pallor.

Laboratory examinations

Laboratory investigations showed three lineage pancytopenia (hemoglobin 66 g/L, WBC count: 2.4 × 10^9

/L, platelet count: 83.0 × 10^9/L). Serum transaminases, bilirubin, and amylase levels were normal. Urine routine, stool routine and occult blood test were negative. Serum tumor markers (AFP, CEA, CA199, and NSE) were all within the normal reference range.

Imaging examinations

Abdominal contrast-enhanced MRI revealed a well-enhanced tumor (size: 2.4 cm × 1.2 cm × 1.2 cm) in the pancreatic tail (Figure 1A), with portal vein invasion (Figure 1B and C), and the presence of many collateral vessels indicative of portal stenosis (Figure 1C). The mass inside the portal vein was enhanced too, suggesting that it was a tumor rather than a thrombus. Splenomegaly was also observed, which was considered as a secondary change. No metastasis was found in other organs, including the lymph nodes, liver parenchyma, and lungs.

FINAL DIAGNOSIS

Histopathologic examination revealed a NEN (G2) with somatostatin receptors 2 (SSTR2) (+) (Figure 2A-D).

TREATMENT

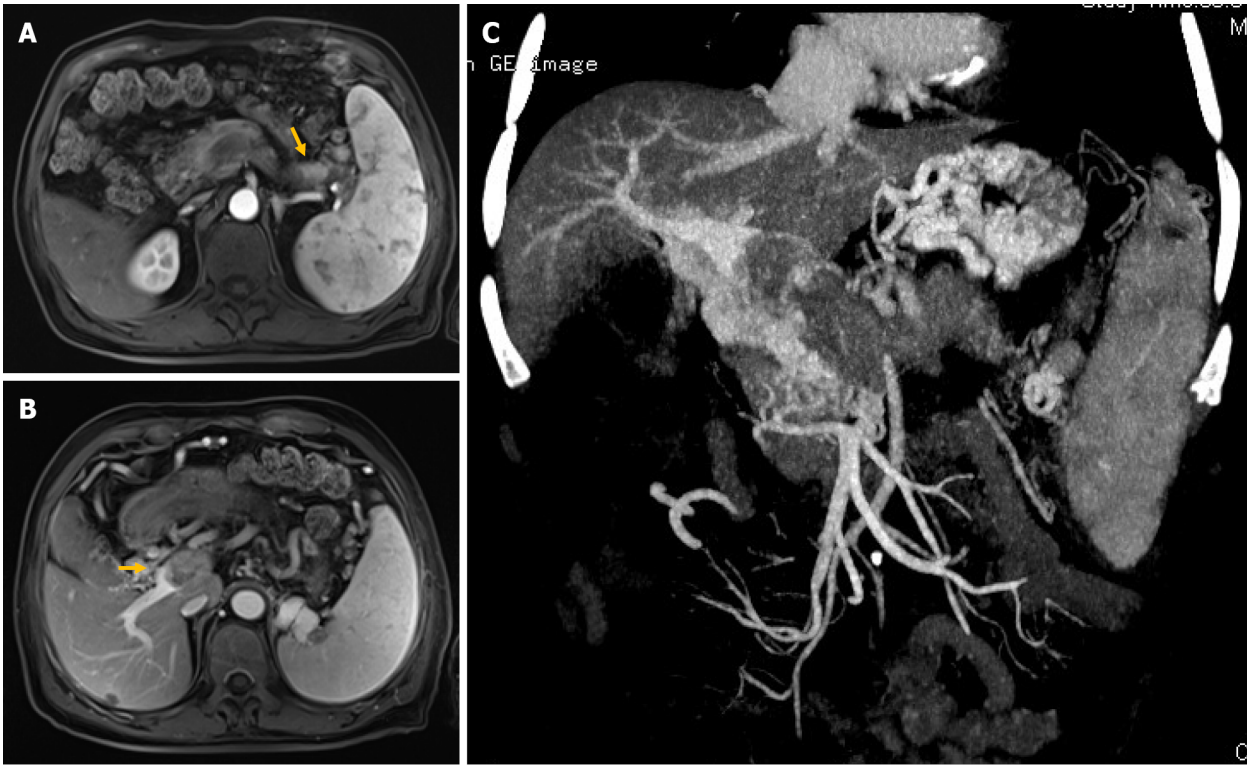
A multidisciplinary team meeting was held to discuss the case. Diagnosis of PNEN with portal vein invasion was highly suspected. Surgical resection was considered risky because of the involvement of adjacent organs and sinistral portal hypertension. Interventional therapy to prevent upper gastrointestinal hemorrhage and chemotherapy were suggested. However, pathological diagnosis *via* EUS-FNA seemed infeasible because of diffuse gastric varices (Figure 3A). The possibility of percutaneous CT/ultrasound-guided fine-needle aspiration biopsy was also excluded since no adequate access was identified. Finally, we performed percutaneous transportal biopsy of the PVTT using a gastroscope biopsy forceps. After percutaneous transhepatic puncture of the right-portal vein under sonographic guidance, 7F "crossover sheath" catheter was introduced into the right-portal vein and the biopsy forceps was inserted into the catheter. Under X-ray guidance, the biopsy forceps was pushed close to the thrombus and 3 samples were obtained (Figure 2E and F; Figure 4; Supplementary material) and fixed immediately. Subsequently, partial splenic embolization (PSE) was performed for treating the gastric varices. After obtaining the histopathologic results, the patient received octreotide long-acting release (LAR) injection. There were no postoperative complications, and the patient was discharged on postoperative day 8. Owing to the high risk of gastric varices hemorrhage, the patient received repeat PSE treatment.

OUTCOME AND FOLLOW-UP

Follow-up gastroscopy performed after one month showed significant alleviation of gastric varices (Figure 3B). Subsequently, the patient regularly received octreotide LAR injection at a local hospital. He experienced weight loss and fatigue for the following 2-4 mo and recovered gradually. He received no chemotherapy or CT/MRI re-examination for some personal reasons. As of 8-mo follow-up, the patient is alive and showed no discomfort.

DISCUSSION

Histological diagnosis is essential for the treatment of patients with PNEN and can be carried out using resection samples, while core biopsies can be performed in patients with advanced disease[4]. ^{68}Ga-DOTATOC-PET-CT, with high sensitivity (92%) and specificity (83%), has been recommended as part of tumor staging, preoperative imaging, and restaging[4]. The EUS features (size and irregular lesion margins) associated with malignancy/aggressiveness of iso- and hypervascular solid pancreatic lesions have also been reported[5]. However, a definitive diagnosis cannot be established based on imaging findings alone. Biopsy can be performed by EUS-FNA/B or percutaneous ultrasound/CT-guided biopsy. Currently, EUS-FNA/B represents the gold standard for diagnosis of pancreatic lesions[6]. This technique provides pathological diagnosis as well as mitotic rate and Ki-67 index which defines grading, with an overall accuracy of approximately 92%[5], leading to its fundamental role in PNENs. However, these approaches may be hampered by gastric varices, poor access windows, target mobility, adjacent critical structures, and skin-to-target remoteness.



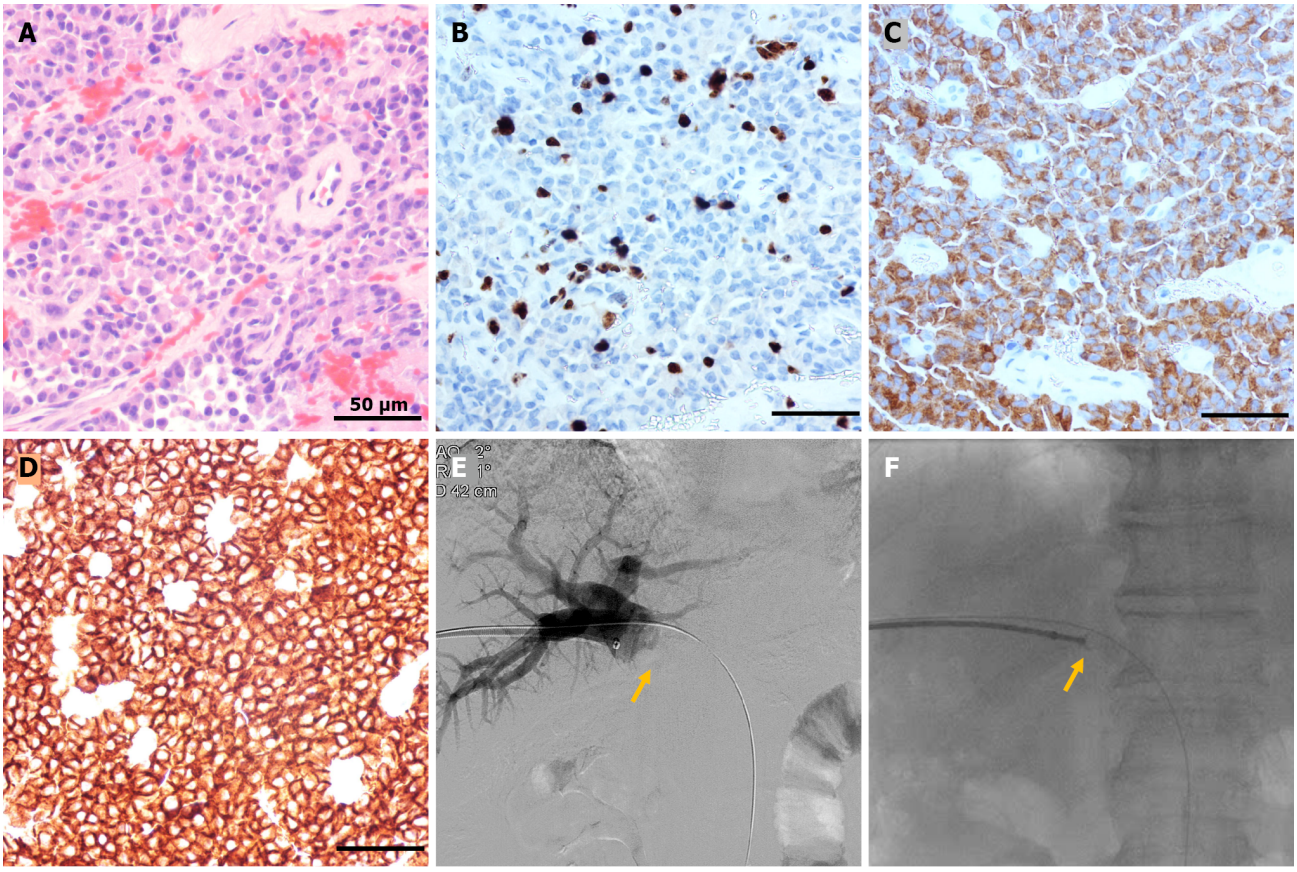
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Figure 1 Representative magnetic resonance imaging and computed tomography image on admission. A: Contrast-enhanced magnetic resonance imaging (MRI) axial view showing a well-enhanced tumor in the pancreatic tail; B: Axial view of contrast-enhanced MRI shows an intravascular filling defect (arrows) occupying the entire portal vein lumen; C: Computed tomography portal venography image showing extensive collateral venous circulation due to portal vein occlusion.

PNENs originate in the endocrine cells of the pancreas, and some of them can secrete specific hormones. Thus, PNENs can be classified as functional PNENs or non-functioning PNENs (NF-PNENs). Due to the lack of symptoms, NF-PENs tend to remain occult until a late stage of disease. In the study by Balachandran *et al*[2], venous tumor thrombus was detected by CT in 29 out of 88 (33%) patients with NF-PNEN. Six of these 29 patients showed invasion of portal vein. Portal venous tumor thrombus (PVTT) is a typical complication of hepatocellular carcinoma and is associated with a poor prognosis[7]. Other causes of PVTT include gastrointestinal and abdominal-pelvic malignant tumors[8]. Pancreatic neoplasms, especially neuroendocrine tumors, represent a possible but uncommon etiology of portal venous invasion[9,10].

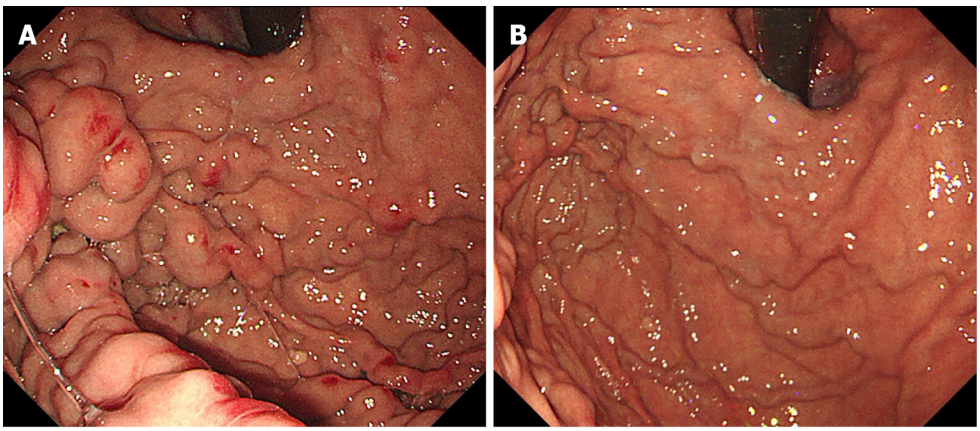
Due to the PVTT in the present case, a gastroscope biopsy forceps-assisted intravascular biopsy was a safe and effective procedure for obtaining samples for histopathology, allowing the initiation of targeted drugs and chemotherapy. Portal vein catheterization has a certain learning curve, and the biopsy device must be introduced into the lesion by transhepatic approach using a large enough catheter (7 Fr). This would lead to a high risk of bleeding due to hepatic tract puncture. To prevent this complication, the puncture tract was embolized with coils, and the patient recovered well without any complications. Clinical use of percutaneous transhepatic portal catheterization was first described in 1970[11,12]. Since then, this has been proven to be a safe technique with few complications[13,14]. It is widely used to control gastroesophageal variceal bleeding[15-17] and to localize gastrointestinal hormone-producing tumors[18-21]. In hormone-producing tumors, percutaneous transhepatic portal catheterization has usually been performed for blood sampling from the portal vein. Here we expanded the use and value of transhepatic portal catheterization for diagnostic purposes.

The use of endoscopic biopsy forceps in intravenous biopsy has been reported. Guirola *et al*[22] reported an intravascular biopsy using a bronchoscopy forceps for facilitating the diagnosis of pulmonary artery intimal sarcoma. Chick JFBreported a transbiliary intravascular ultrasound-guided diagnostic biopsy of an inaccessible pancreatic head mass using core biopsy needle[3]. Sherk *et al*[23] reported a single-center retrospective analysis of 36 patients who underwent transvenous biopsy using cutting biopsy forceps; one of the patients in their study underwent a PVTT biopsy *via* transhepatic portal vein access, which is similar as our work. Apart from this, the use of biopsy forceps in portal vein biopsy has rarely been reported, let alone gastroscope biopsy forceps. Dodd GD III and Vilana R both performed Doppler-guided percutaneous portal vein thrombi biopsy using aspiration needle[24,25]. The potential side effects of percutaneous portal vein sampling include severe biliary or vascular injury. The potential complications include hepatic arterial or portal venous bleeding, bile duct laceration, and



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Figure 2 Morphology and immunohistochemistry image of the tumor and radiograph from portography. A: Hematoxylin and eosin stained section of the tumor; B: Immunohistochemistry staining for Ki-67 showed Ki-67 index of 5%-8%; C and D: Immunohistochemistry staining for synaptophysin (C) and somatostatin receptors 2 (D) were positive (magnification × 400); E: Portography showing a filling defect within the intravascular space (arrows) in the main portal vein; F: Intravascular biopsy using gastroscope biopsy forceps assisted by a 7-F guide catheter located in the right portal vein (arrow).

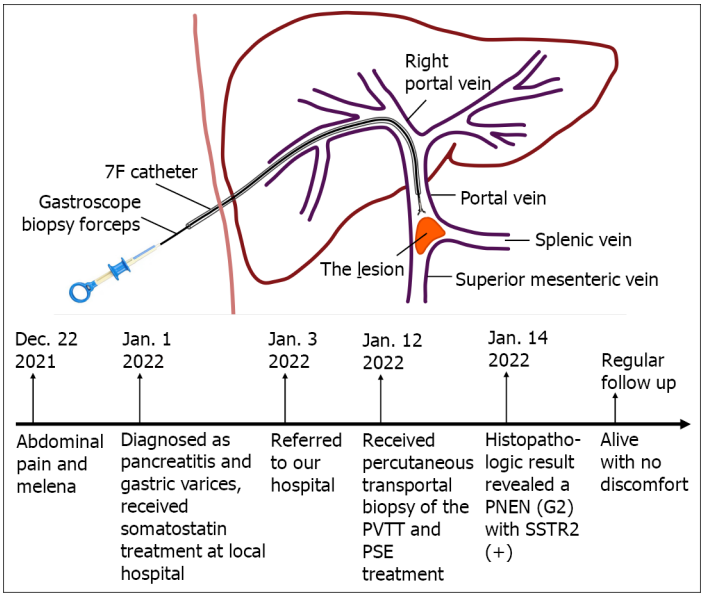


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Figure 3 Endoscopic image on admission and 1 mo later. A: Endoscopic image before partial splenic embolization (PSE) showing diffused gastric varices; B: Endoscopic image after PSE showing alleviation of gastric varices.

formation of biliary-vascular fistula, arterio-venous fistula, or pseudoaneurysm. EUS-FNA of portal venous thrombi represents an alternative approach which might overcome these limitations. Compared with fine needle aspiration, our method may help avert the need for repeated liver puncture, and reduce the risk of injury.

Currently, dual-tracer (^{68}Ga-DOTATOC and ^{18}F-FDG-)-PET/CT scan is a powerful imaging modality for the diagnosis and evaluation of PNET[26]. We did not perform the PET/CT scan owing to the patient's financial constraints. Moreover, the final therapeutic choice for the inoperable tumor would



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Figure 4 Schematic illustration of the procedure and timeline. A: Schematic illustration of the procedure for percutaneous transhepatic intraportal biopsy using a gastroscope biopsy forceps; B: Timeline. PNEN: Pancreatic neuroendocrine neoplasm; SSTR2: Somatostatin receptors 2; PVTT: Portal vein tumor thrombosis; PSE: Partial splenic embolization.

not have changed regardless of the tumor stage. The validity of our method was uncertain prior to this case. Lack of malignant cells in the specimen obtained from a malignant thrombus may lead to a false-negative diagnosis. We tried to prevent false-negative result by obtaining samples for 3 times. Our positive result suggests that tumor cells can be obtained from the PVTT. Our work suggests that the gastroscope biopsy forceps of an appropriate size and function is a suitable substitute to obtain biopsy in the portal vein system. There is still a need for well-designed clinic trials, but this procedure may improve the diagnosis of portal vein thrombosis and related diseases.

CONCLUSION

Percutaneous transhepatic intraportal biopsy is valuable for the diagnosis of portal thrombosis and related diseases. Using gastroscope biopsy forceps through the sheath for tissue sample appears to be safe and effective, and can be performed in the context of other interventional procedures. More clinical trials are required to verify the safety and efficacy of this technique.

FOOTNOTES

Author contributions: Wang GC drafted the manuscript and collected data; Huang GJ performed the operation; Zhang CQ guided the operation; Ding Q guided the operation and revised the manuscript; all authors have approved the final draft submitted.

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ORCID number: Guang-Jun Huang 0000-0001-7138-349X; Chun-Qing Zhang 0000-0001-8711-1579; Qian Ding 0000-0002-2301-8487.

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Editorial Board Member of *World Journal of Gastroenterology*, Ragesh Babu Thandassery, MD, Assistant Professor, Doctor, Department of Medicine, Central Arkansas Veterans Healthcare Systen, Little Rock, AR 72205, United States. doc.ragesh@gmail.com

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REVIEW

Predictors of early and late hepatocellular carcinoma recurrence

Riccardo Nevola, Rachele Ruocco, Livio Criscuolo, Angela Villani, Maria Alfano, Domenico Beccia, Simona Imbriani, Ernesto Claar, Domenico Cozzolino, Ferdinando Carlo Sasso, Aldo Marrone, Luigi Elio Adinolfi, Luca Rinaldi

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Riccardo Nevola, Ernesto Claar, Internal Medicine and Hepatology Unit, Ospedale Evangelico Betania, Naples 80147, Italy

Corresponding author: Riccardo Nevola, MD, PhD, Doctor, Department of Advanced Medical and Surgical Sciences, University of Campania "Luigi Vanvitelli," Piazza Miraglia, Naples 80138, Italy. riccardo.nevola@unicampania.it

Abstract

Hepatocellular carcinoma (HCC) is the most frequent liver neoplasm, and its incidence rates are constantly increasing. Despite the availability of potentially curative treatments (liver transplantation, surgical resection, thermal ablation), long-term outcomes are affected by a high recurrence rate (up to 70% of cases 5 years after treatment). HCC recurrence within 2 years of treatment is defined as "early" and is generally caused by the occult intrahepatic spread of the primary neoplasm and related to the tumor burden. A recurrence that occurs after 2 years of treatment is defined as "late" and is related to de novo HCC, independent of the primary neoplasm. Early HCC recurrence has a significantly poorer prognosis and outcome than late recurrence. Different pathogenesis corresponds to different predictors of the risk of early or late recurrence. An adequate knowledge of predictive factors and recurrence risk stratification guides the therapeutic strategy and post-treatment surveillance. Patients at high risk of HCC recurrence should be referred to treatments with the lowest recurrence rate and when standardized to combined or adjuvant therapy regimens. This review aimed to expose the recurrence predictors and examine the differences between predictors of early and late recurrence.

Key Words: Hepatocellular carcinoma; Early recurrence; Late recurrence; Predictors; Liver transplant; Liver resection; Thermal ablation

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Core Tip: Hepatocellular carcinoma is burdened by a high rate of both early and late recurrence. The knowledge of the predictive factors of recurrence and its risk stratification should allow optimization of the management of the patient with hepatocellular carcinoma.

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INTRODUCTION

Liver cancers represent the fifth neoplasm by incidence and the fourth cause of cancer-related deaths worldwide, with a higher incidence in men than in women[1] and an epidemiological distribution that results from the variations of etiological factors for liver disease (with a current reduction in viral hepatitis and an increase in metabolic etiologies)[2,3]. Hepatocellular carcinoma (HCC) accounts for about 90% of liver cancer cases, with an increasing incidence of 75% from 1990 to 2015[1,4]. The onset of HCC generally follows chronic liver damage resulting in the development of fibrosis and especially liver cirrhosis, although cases of HCC in the absence of liver damage are possible and are generally related to aflatoxin exposure[5].

The main risk factors for HCC are represented by: liver cirrhosis and chronic hepatitis; chronic hepatitis B virus (HBV) infection (with or without hepatitis D virus)[6,7] or hepatitis C virus (HCV)[8,9]; chronic alcohol abuse; metabolic syndrome[10]; diabetes mellitus[11]; and obesity[12]. In particular, 1% to 8% of patients with liver cirrhosis develop HCC annually[13]. Low platelet count, severe portal hypertension, certain comorbidities (*e.g.*, obesity, diabetes mellitus), cigarette smoking, concomitant alcohol consumption, older age and male sex are factors closely associated with the development of HCC in patients with liver cirrhosis[13-17].

The mortality rate of HCC patients, although improving, still appears to be extraordinarily high[18]. An early diagnosis and an appropriate therapeutic approach contribute to higher overall and disease-free survival rates. In particular, when diagnosed at an early stage, HCC can be effectively treated by liver transplantation (LT) or loco-regional techniques, including liver resection (LR) and radio-frequency ablation (RFA). Optimal treatment choice is based on both tumor burden and residual liver function[19-21]. HCC recurrence rates after LT accounts for about 13% of cases[22], and these rates appear significantly higher after loco-regional treatment, reaching 70% of cases at 5 years[4]. The recurrence of the neoplasm may reflect both the presence of intrahepatic metastases ("true" or "early" recurrence) and the development of de novo tumors ("late" recurrence). A 2-year cutoff is generally used to define the two entities[4].

There are numerous factors (related to the patient, the tumor and the type of treatment) able to predict the risk of recurrence after loco-regional treatment or LT. Although many predictors are common to early and late recurrence, other factors may be exclusive to one form due to the different pathogenesis. An adequate recurrence risk stratification would allow personalization of the therapeutic strategy for each patient, optimization of the surveillance program and ideally encourage the study, validation and use of adjuvant therapy in high-risk patients.

The aim of this review was to examine the predictors of HCC recurrence after LT and loco-regional treatment and discuss the differences between predictors of early and late recurrence.

STRATEGIES OF HCC RADICAL TREATMENT

In order to define the most appropriate therapeutic strategy able to maximize outcomes and reduce the risk of recurrence, HCC requires appropriate staging that considers cancer-related factors and residual liver function. The Barcelona Clinic Liver Cancer (BCLC) staging system is the most used to predict patient prognosis and establish treatment allocation[4]. It divides patients into 5 categories (0, A-D) based on tumor characteristics (uni- or multifocality, vascular invasion, size and extrahepatic spread) and liver function (assessed by Child Pugh score).

"Very early HCC stage" (BCLC 0) is defined as patients with preserved liver function and carcinoma in situ (single HCC lesion < 2 cm without vascular invasion), whereas "early stage" (BCLC A) is defined as patients with preserved liver function and a single lesion > 2 cm or 3 lesions with diameters < 3 cm. Patients at BCLC stage 0 and A are optimal candidates for a radical therapeutic strategy (LT, ablation, surgical resection). Patients in more advanced states (BCLC B-D) are candidates for palliative (chemoembolization, systemic therapy) or supportive treatment[4,21].

Due to the excellent long-term outcomes achievable, LT is considered the gold standard for the treatment of HCC[22,23]. It is indicated as the first-line therapy in patients with HCC who meet the Milan criteria (single tumor ≤ 5 cm or multiple tumors as ≤ 3 nodules size ≤ 3 cm) without vascular invasion and/or extrahepatic involvement[24]) but are not eligible for LR[4]. Recent evidence also suggests that patients beyond the Milan criteria could be reconsidered for LT after adequate downstaging of the tumor[25]. LR is the treatment of choice in HCC patients with or without cirrhosis with well-preserved liver function, solitary tumors and clinically mild portal hypertension (hepatic vein to portal system gradient ≤ 10 mmHg)[4,26], with the possibility (not standardized yet) to extend the indication to multicentric tumors or to tumors > 2 cm[4]. It is still one of the main local-regional curative-intent treatments, with a 5-year survival between 40% and 70%[27].

Thermal ablation with RFA or microwaves is the standard of care for HCC patients with BCLC 0 and A stage not suitable for surgery (LT or LR) or as an alternative to LR in very early HCC stage with favorable localization also in patients eligible for surgery[4]. Thermoablation is also indicated as a neoadjuvant therapy in patients who are candidates for LT (pre-transplant) in order to reduce the risk of recurrence. Selected patients with intermediate HCC-BCLC B (solitary > 3 cm or > 3 nodules < 3 cm or advanced liver failure not clinically decompensated) can be reasonably treated with RFA, even if medium- and long-term outcomes appear worse than patients with HCC BCLC 0 or A[28].

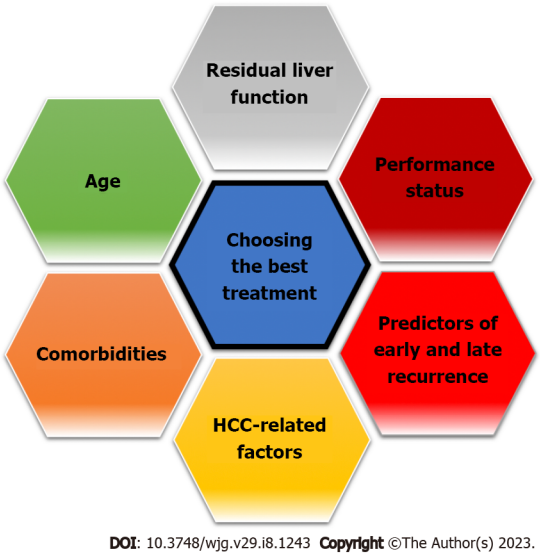
Transarterial chemoembolization (TACE) is the most widely used treatment for unresectable HCC and offers significant overall survival advantages over best supportive care[4], although it has no clear curative purposes and cannot be considered a radical approach. In particular, the survival rates after TACE are 70.3% at 1 year, 51.8% at 2 years, 40.4% at 3 years and 32.4% at 5 years[29]. The rationale of the technique is based on the intermediate HCC intense arterial neo-angiogenic activity and consists in the intra-arterial administration of cytotoxic agents (doxorubicin or platinum derivatives) followed by the embolization of peritumoral vessels inducing a cytotoxic and ischemic effect of the tumor mass. Combination treatments (*e.g.*, TACE + RFA) and neoadjuvant or adjuvant systemic treatments are currently being studied.

EARLY OR LATE RECURRENCE

The recurrence of HCC represents a relevant clinical issue, affecting up to 70% of patients undergoing curative treatment[4]. Although many factors are involved in the decision-making process of the best possible treatment (Figure 1), the assessment of baseline risk recurrence significantly influences the choice of treatment type and its timing. Moreover, the treatment of a relapse is generally more complex than the treatment of the first lesion, due to the change in anatomical or functional liver conditions and a more advanced age. Patients at high risk of early recurrence are candidates for close surveillance and potentially adjuvant therapy, which has not yet been standardized[30,31].

The "true" recurrence (secondary to the presence of occult intrahepatic metastases) is generally "early" and accounts for more than 70% of tumor recurrence. It derives from an intrahepatic dissemination of the neoplasm through the portal circulation. A 2-year cutoff is used to distinguish it from late recurrence, generally resulting from de novo development of the neoplasm. However, this cutoff remains arbitrary and not universally accepted. Yamamoto *et al*[32], for example, identified 17 mo as the best cutoff to distinguish early and late recurrence on a cohort of 252 patients with recurrence of HCC after hepatectomy. Previously Hayashi *et al*[33] had hypothesized a 1 year cutoff instead. A further shorter observation period has recently been hypothesized by Xing *et al*[34] who identify 8 mo as the ideal threshold to define a recurrence of HCC as early or late. Although the temporal criterion is used to distinguish a pathogenesis related to the presence of occult intrahepatic metastases or to a de novo neoplasm, a precise differentiation would require the study of recurrence clonality by genetic/genomic analyzes[35].

The two types of recurrence are two distinct entities associated with different risk factors. Early recurrence is associated with tumor-related factors, whereas late recurrence is related to underlying liver disease. They have a substantially different clinical and biological profile and are burdened by different morbidity and mortality. In fact, an early recurrence represents a significant negative prognostic factor and has a far greater impact on overall survival than a late recurrence[32,36]. Xing *et al*[34] demonstrated that patients with early recurrence showed a median of survival free from cancer of 8.4 mo (7.5-10.0 mo) compared to a median of 21.3 mo (17.9-23.8 mo) for patients with late recurrence. In particular, an early recurrence, mainly determined by the aggressiveness of the primary tumor, is characterized by larger dimensions, higher rates of multifocality and intrahepatic spread, higher probability of vascular invasion and higher levels of alpha-fetoprotein (AFP) compared to a recurrence that develops later[36]. Conversely, late recurrence is generally related to etiology and cirrhosis, risk factors for hepatocarcinogenesis, and not to the primary tumor[37].



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Figure 1 Determinants of therapeutic decision making for hepatocellular carcinoma. HCC: Hepatocellular carcinoma.

EARLY RECURRENCE PREDICTIVE FACTORS

The factors affecting the possibility of early HCC relapse after LT or loco-regional treatment can be classified into three categories: those related to the tumor (size, number of nodules, differentiation, oncological markers); to the patient (*e.g.*, age, comorbidity, liver function, possible viral load, presence and activity of hepatitis, presence and activity of liver cirrhosis); and treatment (type of treatment, margins, characteristics of resection) (Table 1)[38,39].

Predictors of early recurrence related to HCC characteristics

Several factors related to the characteristics of HCC are able to predict the risk of early recurrence (Table 1). This risk is closely related to the tumor burden and its aggressiveness, which increases the probability of occult intrahepatic spread and affects the radicality of the treatment[40].

Preoperative radiological imaging can already provide first indications about the risk of relapse. In this regard, the size of the tumor represents a relevant predictor of the risk of early recurrence. Jung *et al*[36] showed that a maximum diameter of HCC greater than 3 cm before a curative LR represented an independent risk factor for early recurrence (defined by the authors as occurring within 1 year of treatment). These results have recently been confirmed by Lee *et al*[41]. Zhu *et al*[42] identified a 4.77-fold higher risk of early recurrence after an LR for cancers with a maximum diameter greater than 2.6 cm compared to those with a smaller maximum diameter[42]. Similarly, the multifocality of HCC seems to be another significant risk factor for early recurrence[41]. Tumor size and number of lesions represents together the aforementioned Milan criteria. As expected, being beyond the Milan criteria represents an independent risk factor for early HCC recurrence[43].

At histological evaluation, the most predictive factors of the risk of early HCC recurrence are the presence of microvascular invasion, the integrity of the capsule and the degree of differentiation. In light of the fact that early recurrence is generally due to intrahepatic dissemination of the neoplasm through the portal circulation, it is not surprising that vascular invasion has been identified many times as an independent predictor of recurrence. If the involvement of the major vessels (*e.g.*, portal vein, inferior vena cava) can be evaluated radiologically and often excludes the possibility of loco-regional treatment for radical purposes, microvascular invasion needs to be evaluated by histopathological analysis of the intraoperative sample. Microvascular invasion, although dependent upon the operator and influenced by possible sampling errors, represents an independent risk factor for early recurrence[34,44,45] affecting approximately one-third of HCC cases that underwent LR[37]. Since a qualitative and/or quantitative classification of microvascular invasion is not yet available, its only presence/absence is generally considered in the histopathological evaluation. However, in this regard, Roayaie *et al*[46] proposed a risk system based on histological features of microvascular invasion that includes invasion of a vessel with a muscular wall and invasion of vessels ≥ 1 cm away from the tumor capsule. According to the authors, this system stratifies patients into three distinct groups with significantly different risks of recurrence and death. In particular, patients with microvascular invasion and the aforementioned risk factors show outcomes (tumor recurrence rate, mortality rate) comparable to patients with macroscopic vascular invasion.

The integrity of the HCC capsule would instead represent a protective factor for the risk of recurrence [42]. The capsule would act as a barrier to the spread and metastasis of cancer cells. This function is lost

Table 1 Predictors of early hepatocellular carcinoma recurrence after curative treatment

Predictors related to HCC characteristics	Ref.	Predictors related to patient characteristics	Ref.	Predictors related to treatment	Ref.
Size		Liver cirrhosis	[37,41]	RFA (*vs* LR)	[41,88,89]
> 2.6 cm	[42]				
> 3 cm	[36,41]				
Multifocality	[41]	Liver insufficiency (CPS, MELD, ALBI)	[37,56-58]	LR:	
				Liver resection margins invasion	[44]
				Liver resection margins < 1 cm	[43,86]
				Non-anatomical resection[1]	[45]
Beyond Milan criteria	[43]	High total bilirubin	[42]	RFA: ablation margins < 1 cm	[91]
Microvascular invasion	[34,36,38,40,44,45]	Male sex	[37]		
Lack of capsule integrity	[34,42]	High PLR	[49,59,60]		
Poor histological differentiation	[36,43,47]	High NLR	[49,60]		
High AFP	[36,41,50]	DAAs therapy			
> 10 ng/mL	[48]	Predictor	[62,63]		
> 32 ng/mL	[45]	Non-predictor	[64-76]		
> 400 ng/mL	[40]	Protective	[77-83]		
High PIVKA-II	[41,52,55]	High viral load in HBV infected patients	[109]		
> 46.0 mAU/mL	[38]				
> 375.5 mAU/mL	[51]				

[1]Available data are contradictory and not conclusive.

AFP: Alpha-fetoprotein; ALBI: Albumin-Bilirubin score; CPS: Child-Pugh score; DAA: Direct antiviral agent; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; LR: Liver resection; MELD: Model for end-stage liver disease; NLR: Neutrophil-to-lymphocyte ratio; PIVKA-II: Protein induced by vitamin K absence or antagonist II; PLR: Platelet-to-lymphocyte ratio; RFA: Radio-frequency ablation.

in non-capsular HCC or those with a ruptured capsule[34,42]. Zhu *et al*[42] showed that non-capsular HCC was associated with a higher rate of poorer differentiation and vascular invasion than capsular HCC.

Histological differentiation is considered an independent risk factor for early HCC recurrence. It is classified according to the Edmonson-Steiner criteria: 1st and 2nd degree correspond to well differentiated neoplasms; and 3rd and 4th degree correspond to poorly differentiated neoplasms. In particular, grades 3 and 4 are related to a significantly increased risk of early recurrence[36,43]. In patients without microvascular invasion, the degree of differentiation seems to be the best histological predictor of the risk of early recurrence and overall survival[47].

In light of the growing evidence of the efficacy in predicting the risk of recurrence, histological evaluation will become crucial in choosing the optimal therapeutic strategy for each patient, allowing the implementation of the stratification of the basal recurrence risk with the postoperative one. Patients at high risk of recurrence should undergo an aggressive surveillance strategy and ideally adjuvant therapy.

Among the predictors of early recurrence, the markers expressed by the neoplasm are certainly the most studied. In particular, AFP, a glycoprotein physiologically produced by the liver and fetus, can increase in pathological conditions, such as liver cancer. In addition to the diagnostic phase, it plays an important role as a predictor and monitoring tool in the context of HCC recurrence. There is almost unanimous agreement on the predictive value of elevated AFP levels for the risk of early HCC recurrence after treatment[36,48,49], but there are no standardized cutoffs pre- and post-treatment.

Jung *et al*[36] showed that high AFP values both pre- and post-hepatectomy were associated with a higher risk of early recurrence (which the authors defined as < 1 year after treatment) but without a standardized threshold value. Using a retrospective data analysis, Kim *et al*[48] showed that preoperative AFP values > 10 ng/mL were a predisposing factor of disseminated HCC recurrence

within 3 mo after hepatectomy for solitary HCC [odds ratio: 5.333; 95% confidence interval (CI): 1.095–25.985]. Fang *et al*[40] showed instead that preoperative AFP > 400 ng/mL correlated independently with the risk of early recurrence. Recently, AFP levels at 12 wk after achieving sustained virological response with direct antiviral agents (DAAs) treatment in chronic HCV patients have also been independently associated with a risk of HCC recurrence[50].

The protein induced by vitamin K absence or antagonist II (PIVKA-II) is an immature form of prothrombin, a cofactor of vitamin K that is synthesized by the liver when the latter is not produced or antagonized. Evaluation of PIVKA-II has been shown to be useful in diagnosing HCC and in stratifying the risk of recurrence[38,41,51,52]. In fact, it acts as a growth factor, promoting cell proliferation and tumor angiogenesis in HCC patients[52,53]. The evaluation of PIVKA-II appears to be complementary to AFP[54]. Low values of both AFP and PIVKA-II are associated with a better prognosis compared to elevated levels of AFP and/or PIVKA-II[55]. Serial measurements of both proteins allow a prompt diagnosis of early recurrence. In fact, pre- and post-treatment levels of PIVKA-II are good predictors of early HCC recurrence[38,41,51] since they are related to greater aggressiveness of the neoplasm, such as the presence of vascular invasion and intrahepatic metastasis of HCC cells[52].

Similarly to what is highlighted for AFP, there is no agreement on a PIVKA-II cutoff that is useful as a guide for assessing the risk of recurrence. In this regard, Wang *et al*[51] showed that in patients with a low risk of recurrence (tumor size < 5 cm, single tumor, absence of satellite lesions, absence of vascular invasions, high degree of histological differentiation, BCLC stage 0-A), a preoperative value of PIVKA-II > 375.5 mAU/mL was the strongest independent prognostic factor for the risk of early recurrence [hazard ratio (HR): 2877; 95%CI: 1524-5429]. Furthermore, patients who expressed high levels of PIVKA-II showed lower 1-year time-to-progression than patients with low levels (54.8% *vs* 20.2%, respectively). More recently, Hong *et al*[38] identified a cutoff of 46 mAU/mL for PIVKA-II for predicting an increased risk of early recurrence.

Predictors of early recurrence related to patient characteristics

Predictors related to patient characteristics are associated with the risk of late recurrence. In fact, they represent the substrate for the risk of hepatocarcinogenesis and generally do not correlate with the aggressiveness of the primary tumor, which is strongly associated with early recurrence instead. However, some studies have shown a certain impact of the patient's basal phenotype on HCC recurrence that occurs in the first 2 years after treatment (Table 1).

In this regard, the presence of liver cirrhosis and the degree of hepatic dysfunction are the factors that most increase the risk of early recurrence after curative treatment[37,41]. In the models used for risk stratification in patients undergoing hepatectomy, the degree of hepatic dysfunction is quantified by the Child Pugh score[56,57], the model for end-stage liver disease[41] or by the Albumin-Bilirubin score[37,58]. The latter in particular seems to have better discriminatory power in this setting since it helps to further stratify stage A of the Child-Pugh score, which includes almost all patients undergoing LR[58]. Total bilirubin levels, an expression of the degree of hepatic dysfunction, also correlate with the risk of early recurrence[42]. Furthermore, according to Chan *et al*[37] male patients undergoing hepatectomy show a higher risk of early recurrence than female patients.

The predictive value on outcomes (mortality and recurrence) of the neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) in HCC patients has been extensively studied. These scores are an expression of the systemic inflammatory response, a known preoperative risk factor for HCC outcomes[49,59,60]. High PLR has been shown to predict lower overall survival (HR: 1.63, 95%CI: 1.34-1.98) and earlier HCC recurrence (HR: 1.52, 95%CI: 1.21-1.91)[59]. Similar results were also highlighted for NLR[49,60]. In addition, both the NLR and PLR were identified as independent risk factors for predicting overall survival and recurrence-free survival (RFS) in HCC patients[59,60].

In recent years, a heated scientific debate has been carried out on the hypothesis of an increased risk of HCC incidence and recurrence in chronic HCV patients treated with DAAs. Unlike interferon (IFN)-based therapeutic regimens, which have been proven to reduce the incidence and recurrence of HCC in cases of sustained virological response[61], some data indicated that after DAA therapy HCC risk may remain high[4,62]. In particular, the retrospective analysis of some patient cohorts revealed an unexpectedly high rate of early HCC recurrence in patients undergoing antiviral treatment with DAAs [62,63]. It has been hypothesized that the rapid fall in viral load induced by DAAs could lead to an altered immune surveillance, promoting the growth of already existing cancer clones[4]. However, the higher number of patients eligible for antiviral treatment with DAAs could influence the apparent raise in early recurrence rates. In fact, due to the manageability and safety profile of these therapeutic regimens, a larger number of patients with advanced liver disease at high risk of HCC (*e.g.*, liver cirrhosis, advanced age, multiple morbidity) have undergone antiviral treatment than those who were candidates for IFN-based schemes. Subsequent prospective studies underlined that although the rates of early HCC recurrence remain high in patients who have obtained viral clearance by DAAs, these rates are comparable to those reported in the literature for patients not treated with these regimens[64-66].

Waziry *et al*[67], in a meta-analysis, concluded that there is no evidence that DAA therapy was associated with higher HCC recurrence and that the available data do not suggest differentiated surveillance pathways for HCC among patients receiving IFN-free therapeutic regimens or receiving DAA treatment. However, the heterogeneity of the studies included in this analysis limited the power of

its results. Although there is no conclusive evidence at the moment in favor or against an increased risk of early HCC recurrence in patients undergoing treatment with DAAs, it is clear that the risk of early HCC recurrence in patients with HCV-related cirrhosis remains high despite viral eradication.

The latest guidelines of the European Association for the Study of the Liver (published in 2018) suggested a more intensive surveillance strategy (3-4 mo imaging intervals for the first 2 years that can be extended to 6-mo intervals thereafter) in this patient setting[4]. However, the updated literature available after the publication of these guidelines almost unanimously confirmed that DAA therapy was not associated with an increase in early HCC recurrence rates and that both recurrence rates and tumor patterns after initiation of antiviral therapy did not differ between patients who received IFN-based or IFN-free therapy[68-76]. On the contrary, there is now increasing evidence of a reduction in the risk of early (as well as late) HCC recurrence after viral eradication achieved by DAA[77-83].

Predictors of early recurrence related to HCC treatment

The choice of treatment for HCC depends on the characteristics of the patient (in particular the residual liver function and the performance status) and the neoplasm characteristics (size, single or multifocal, vascular invasion, distant metastasis) as well as the indication and eligibility for LT.

In terms of recurrence risk, no conclusive data are available in the literature about which type of loco-regional treatment is more advantageous. In the absence of liver cirrhosis, LR probably offers the best prognostic prospects, with an early recurrence rate of approximately 10%[36]. Including patients with liver cirrhosis, the early recurrence rates of post-hepatectomy HCC reported in the literature range from 25% to 50%[34,84,85]. Metachronous HCC, new postoperative lesions and intrahepatic metastasis from primary HCC are in fact relatively common following hepatectomy[42]. In this regard, the resection margins after hepatectomy appear to be closely related to the risk of recurrence. In particular, neoplastic invasion of the resection margins represents a significant predictor of the risk of recurrence[44]. A resection margin < 1 cm also seems to be an independent risk factor for early HCC recurrence[43,86]. Some evidence also suggests that a non-anatomic hepatic resection (resection or enucleation without regard to sectoral structure) could lead to an increase in early recurrence rate[45]. In fact, an anatomic resection (resection of the entire liver parenchymal tissue supplied by the portal venous system draining the HCC tissue, independent of margin length) could hinder cell dissemination, reducing tumor cell flow throughout the portal circulation and consequently reduce the risk of intrahepatic metastasis and improve RFS. However, the absence of randomized clinical trials comparing anatomical and non-anatomical hepatic resection do not provide conclusive indications[87].

As previously mentioned, a maximum tumor diameter greater than 2.6 cm (according to Zhu *et al*[42]) or 3.0 cm (according to Jung *et al*[36]) before a curative LR is predictive of a high rate of early recurrence. The risk of early HCC recurrence with a diameter greater than 2.6 cm appears 4.77-fold higher than neoplasms with a diameter ≤ 2.6 cm[42]. Furthermore, high preoperative total bilirubin levels also correlate with an increased risk of recurrence[42]. In particular, this risk would be increased by 6% for every 1 mmol/L increase in preoperative bilirubin. Chan *et al*[37] in a large multicenter study provided pre- and post-hepatectomy risk stratification models (ERASL-pre and ERASL-post, respectively) with the ability to identify three early recurrence risk classes, with a 2-year malignancy free survival rate of 64.8%, 42.5% and 20.7% in low, intermediate and high risk patients, respectively. In these models male sex, large tumor size, multifocal tumor, high Albumin-Bilirubin score and high serum AFP represented the factors closely related to early recurrence after LR. Ideally, these models, once validated, would optimize the therapeutic strategy for patients with high baseline risk of early recurrence after hepatectomy (revaluating the possibility of LT or providing adjuvant therapy) and intensify the surveillance program.

Compared to LR, RFA treatment shows significantly higher recurrence rates[41,88,89]. In particular RFA results in shorter overall survival and time to recurrence (5-year overall survival of 71.1%, 5-year recurrence time of 63.8%) than LR (5-year overall survival of 61.1%, 5-year recurrence time of 71.7%) [88]. The HR for death (0.84, 95%CI: 0.74-0.95) and recurrence (0.74, 95%CI: 0.68-0.79) was significantly lower in patients undergoing LR than in those undergoing RFA. Recently, Lee *et al*[41], through a retrospective study comparing the two methods, showed that in patients with BCLC stage A HCC treatment with RFA was associated with a 1.8 times higher probability of HCC recurrence than hepatectomy (95%CI: 1541-2216). While confirming higher overall survival rates after LR compared to RFA, other data were unable to demonstrate significant differences in RFS between the two methods [90]. Finally, similar to what was shown for LR, an ablation margin ≥ 10 mm away from the tumor lesion guarantees better outcomes and a lower rate of recurrence compared to an ablation margin ≥ 5 mm and < 10 mm[91].

PREDICTIVE FACTORS OF LATE RECURRENCE

Conventionally, HCC recurrence is defined as late when it occurs 2 years after treatment of the primary tumor. Late recurrence is generally related to de novo development of the neoplasm and does not depend on the characteristics of the previous tumor. About 90% of late relapses consist in exclusively

intrahepatic localization, whereas the remaining cases show both intrahepatic and extrahepatic localizations[92]. Therefore the risk factors are mainly related to underlying liver disease (Table 2).

Predictors of late recurrence related to patient characteristics

Among the predictive factors of late recurrence related to the basal characteristics of the patient, it is easily understood that the main predictive factor is the presence of liver cirrhosis, a fertile substrate for hepatocarcinogenesis[93-96]. In particular, the presence of liver cirrhosis increases the risk of late HCC recurrence by 3 or 4 times[41,93]. In fact, liver cirrhosis is a pre-malignant condition that due to an accelerated hepatocyte turnover induced by the chronic inflammatory state promotes the accumulation of gene aberrations and cell transformations. The high rate of gene errors and the subsequent uncontrolled cell proliferation therefore favor the development of HCC[97]. However, patients with liver cirrhosis undergoing curative treatment for HCC show a late recurrence rate significantly higher compared to de novo incidence rate in patients with no history of prior HCC[41]. This implies that the presence of liver cirrhosis is not the only factor that elicits the cancer risk in patients with late recurrence.

As highlighted by Lee *et al*[41], Xu *et al*[92] and Yang *et al*[93], the age and sex of the patient also contribute to the late recurrence risk stratification. Older and male patients show an increased risk of late HCC recurrence after curative treatment. The sex difference in the late recurrence rate could be explained by the potential protective role of estrogen on the development of HCC[98,99], resulting in more than 3 times higher probability of late recurrence in men compared to women[93].

The predictive role of liver stiffness (LSM) in the risk of HCC occurrence is well documented in the literature[100,101]. Since occurrence and late recurrence show similar pathogenesis (de novo hepatocarcinogenesis), it seems reasonable to believe that LSM can also represent a risk factor for late recurrence. In this regard, Jung *et al*[102] had already highlighted how the presence of liver cirrhosis assessed by LSM ≥ 13.5 kPa was associated with an increased overall risk of recurrence. More recently, Marasco *et al*[43] demonstrated that LSM but even more the splenic stiffness (surrogate for the degree of portal hypertension) were associated with an increased risk of late HCC recurrence. Late RFS was significantly different according to the splenic stiffness cutoff of 70 kPa.

These data find further evidence in the literature. Fang *et al*[40] in fact showed how the splenic volume (evaluated through automated volumetry software from preoperative computed tomography images) correlated independently with the probability of HCC recurrence 2 years after hepatic resection. In particular, for each 1 mL increase in splenic volume, the risk of late recurrence increased by 0.3%. In addition, patients with high splenic volume (> 165 mL) showed a lower 5-year RFS (5-year RFS 36%) than patients with low splenic volume (< 165 mL, 5-year RFS 71%). The correlation between stiffness/splenic volume and the risk of late HCC recurrence probably comes from the close association with the severity of liver disease and portal hypertension, both involved in hepatocarcinogenesis. The correlation that some studies showed between the risk of late recurrence and platelet counts or the presence of esophageal varices, which are also surrogates of the degree of portal hypertension and severity of liver damage[43,103,104], is therefore easily explained.

In patients with chronic HBV infection, the presence of viral replication increases the risk of late recurrence[105-108]. In particular, patients undergoing LR for HCC with high preoperative HBV-DNA levels show lower median overall survival and RFS compared to those with low viral load[106]. Patients with high viremia are characterized by higher Ishak inflammatory and fibrosis scores, favoring hepatocarcinogenesis[105]. The liver inflammatory activity induced by the high viral load may lead to necrosis and regeneration of hepatocytes, increasing the rate of gene errors and neoplastic transformation.

Recently, it was hypothesized that a high viral load (HBV-DNA > 10^4 copies/mL) was a risk factor also of early recurrence[109]. In fact, high preoperative levels of HBV-DNA increased the risk of microvascular invasion by about 40%[110]. In patients with high preoperative viral load, the initiation of antiviral treatment after surgical resection reduced both early and late HCC recurrence rates[106,109,110]. However, a similar effect on late recurrence rates was also found in patients with low preoperative viral loads[111]. Despite comparable efficacy in obtaining virological response, recent evidence suggested that tenofovir disoproxil fumarate (TDF) treatment was associated with a significantly lower risk of both HCC occurrence[112] and early and late recurrence[113] compared to treatment with entecavir (ETV). In particular, the HCC recurrence rate at 5 years from surgical resection was 33.6% in patients treated with TDF and 44.5% in those treated with ETV[113]. Unlike ETV, TDF induced the synthesis of high serum levels of IFN-lambda 3 (IFN-λ3)[114], which has been shown to exert a strong antitumor activity[115,116]. The antitumor effect of IFN-λ3 induced by TDF would therefore be additive to the capacity of breaking down viral replication and turn off necroinflammatory activity, which is common to both nucleoside (ETV) and nucleotide (TDF) analogs.

Increasing evidence is also available on the predictive role of hepatitis B surface antigen (HBsAg) levels on the rate of late HCC recurrence. In patients undergoing LR, preoperative HBsAg levels > 200 IU/mL were independent predictors of late recurrence (HR: 1778)[84,103]. If the role of the predictor is well defined, there is nevertheless a significant heterogeneity of the cutoffs used. Huang *et al*[86] in fact highlighted how in patients with low viral load the risk for HCC recurrence significantly increased with HBsAg levels ≥ 1000 IU/mL (5-year RFS rate of 46.1% in HBsAg ≥ 1000 IU/mL group *vs* 34.1% in HBsAg < 1000 IU/mL group). According to Sohn *et al*[107], HBsAg levels ≥ 4000 IU/mL were associated

Table 2 Predictors of late hepatocellular carcinoma recurrence after curative treatment

Predictors related to HCC characteristics	Ref.	Predictors related to patient characteristics	Ref.	Predictors related to treatment	Ref.
Size > 5 cm	[92]	Liver cirrhosis	[32,41,43,84,93-96]	RFA (*vs* LR)	[41,88,117-119]
Multifocality	[92,93]	Old age	[41,92]		
Microvascular invasion	[92]	Male sex	[93]		
AFP > 400 μg/L	[94]	LSM	[43,102]		
		Portal hypertension			
		SSM (> 70 kPa)	[43]		
		Splenic volume (> 165 mL)	[40]		
		Platelet count	[43,103,104]		
		Esophageal varices	[43,103]		
		HBV infection			
		High viral load	[105,108]		
		Low viral load	[111]		
		High HBsAg levels	[84,86,103,107]		

AFP: Alpha-fetoprotein; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; LR: Liver resection; LSM: Liver stiffness; RFA: Radio-frequency ablation; SSM: Splenic stiffness.

with late recurrence after curative resection in HBV-related HCC.

Predictors of late recurrence related to HCC treatment

Several studies suggested that RFA treatment increases the risk of early and late recurrence (HR: 1872; 95%CI: 1290-2717) compared to LR[41,88,117-119]. Furthermore, the overall survival at 3 years and 5 years of patients undergoing LR appeared significantly higher than patients undergoing RFA[117,118]. In particular, the 5-year overall survival rates were 80% *vs* 66%, and 5-year RFS rates were 48% *vs* 18% for LR and RFA groups, respectively[118]. Because the choice of treatment is influenced by both tumor- and liver disease-related factors, some authors hypothesize that the correlation between RFA treatment and the risk of late recurrence could reflect both. In fact, patients undergoing RFA are generally older, have a greater number of comorbidities and a worse degree of liver function than patients undergoing LR. As noted above, these factors are closely related to the risk of both late and early recurrence. Therefore, the higher risk of late recurrence in patients who received RFA compared to those treated with resection could depend on more severe underlying liver disease.

Predictors of late recurrence related to HCC characteristics

Some evidence suggested (which was different from what was expected[95,96]) that factors related to tumor burden (size, multifocality) determined an increase in the risk of late and early recurrence[45,93,105]. Recently, Xu *et al*[92] confirmed in a multicenter retrospective analysis of patients who underwent curative LR for HCC that multifocality, dimensions greater than 5 cm and the presence of satellite nodules or vascular invasion (macroscopic or microscopic) represented independent risk factors of late recurrence. The presence of multifocal HCC at baseline increased the risk of a late recurrence after curative treatment by more than 3 times (HR: 3766, 95%CI: 2287–6201)[93]. In this regard, Cheng *et al*[94] also showed that pre-hepatectomy AFP levels > 400 ug/L were related to the risk of late (as well as early) recurrence. Given the dichotomy between early and late recurrence, these factors should correlate with an increased probability of occult intrahepatic dissemination (and therefore early recurrence) rather than an increased risk of hepatocarcinogenesis (late recurrence). These data suggest that the dichotomy between early and late recurrence is probably not so clear-cut, and pathogenetic mechanisms and clinical features may overlap. Therefore, the temporal criterion alone is not able to discriminate the pathogenesis and aggressiveness of a recurrence of HCC with certainty.

LIVER TRANSPLANT AND HCC RECURRENCE RISK

LT represents the most radical approach for HCC in patients with liver cirrhosis and is able to treat

simultaneously the neoplasm and the underlying liver disease while minimizing the risk of both early and late recurrence when candidate selection is adequate[120]. In fact, a therapeutic strategy that provides for early listing for transplantation and a loco-regional bridging therapy in patients with HCC at high risk of recurrence has proven to be extremely valid and associated with excellent long-term outcomes[121]. The presence of occult extrahepatic dissemination and the persistence of the cause of liver damage (*e.g.*, HBV infection or HBV/hepatitis D virus coinfection) account for the residual risk of early and late HCC recurrence, respectively. Overall, the estimated HCC recurrence rate in patients undergoing LT is between 12% and 20%[22,122,123].

The most important predictor of the risk of early post-transplant recurrence is certainly the tumor burden. Size and number of lesions closely correlate with this risk, although not in a linear way. In fact, in multifocal HCC, starting from three or more lesions, the increase in the risk of recurrence appears to be attenuated[124]. Conversely, this risk is proportional to the size of the tumor. In particular, the recurrence rate increases by 36% for each additional centimeter of HCC diameter[125]. The microvascular invasion is another determining factor in the risk of recurrence and HCC-related death [126,127]. Its presence increases the risk of recurrence by approximately 2.4 times and significantly reduces the 5-year rates of RFS (44% *vs* 64% in the absence of microvascular invasion)[126]. Similarly, the histological finding of poorly differentiated (grade 3 or 4) HCC determines an increased risk of recurrence (39.3% *vs* 13.0% for grade 1 and 2 tumors) and reduction of 5-year RFS (39.9% *vs* 57.7%)[127]. In this setting, the prognostic role of AFP is also relevant with an inverse relationship with the post-transplant survival rate[128]. An increase in AFP greater than 7.5 ng/mL per month is associated with the presence of microvascular invasion and is predictive of post-transplant recurrence[129].

Beyond the characteristics and aggressiveness of the tumor, the etiology of liver disease also affects the rate of recurrence, especially late recurrence. In particular, the highest recurrence rate was found in patients with chronic HBV infection (18%) compared to other etiologies (11%, 10% and 8% for HCV, alcoholic liver disease and nonalcoholic steatohepatitis, respectively). In this regard, preliminary evidence suggested that an increase in RFS for HCC patients following LT can be obtained by administering anti-HBV prophylaxis and/or anti-HBV immunoglobulins[130].

Finally, some comorbidities may also influence the risk of recurrence in HCC patients undergoing LT. In particular, obese patients show a significantly higher frequency of microvascular invasion and recurrence rates and lower RFS than normal weight patients[131,132]. This correlation could be explained by a more pronounced tumor neoangiogenesis in obese patients, secondary to the increased expression of vascular endothelial growth factor. Furthermore, in obese patients the reduction of adiponectin levels and the simultaneous increase of leptin induced a pro-oncogenic state and stimulated neoplastic proliferation[131,132].

The central theme in managing the risk of recurrence is the selection of patients eligible for LT. Currently, the strongest evidence identifies the Milan criteria as the best strategy to optimize the selection of LT candidates, stratifying the risk of early recurrence, strongly correlated to tumor size and number of focal lesions[22]. These criteria suggest the presence of single tumors ≤ 5 cm or multiple tumors ≤ 3 nodules sized ≤ 3 cm, without vascular invasion and/or extrahepatic involvement as boundaries for transplant eligibility[24]. The application of these criteria guarantees a post-transplant survival rate comparable to that of patients undergoing transplants for non-neoplastic causes[133]. On the other hand, patients undergoing organ transplantation beyond the Milan criteria show significantly higher HCC recurrence rates than patients within these criteria[22].

However, growing evidence suggests that these criteria, developed in 1996, may be excessively restrictive to date, leading to the exclusion of a subgroup of patients who could benefit from transplantation[120]. Yao *et al*[134] showed that expansion of the tumor size limits (solitary tumor ≤ 6.5 cm or ≤ 3 nodules with the largest lesion ≤ 4.5 cm and total diameter ≤ 8 cm-UCSF criteria) does not adversely impact survival post-transplant. Subsequently, the same authors validated these criteria, confirming a 5-year RFS rate of 81%[135]. The extension of the Milan criteria suggested by Yao *et al*[135] made the 5%-20% of previously excluded patients eligible for LT, guaranteeing comparable long-term survival rates. Also Mazzaferro *et al*[124] attempted to overcome the previous limits through the up-to-seven criteria: HCC with seven as the sum of the size of the largest tumor (in centimeters) and the number of tumors. They showed that in the absence of microvascular invasion patients who met these criteria demonstrated survival rates comparable to patients who met the original Milan criteria (5-year overall survival of 71.2%). Conversely, the presence of microvascular invasion doubled the likelihood of recurrence and significantly reduced the overall post-transplant survival rates in these patients (up-to-seven patients 5-year overall survival of 53.6% *vs* Milan criteria patients 5-year overall survival of 73.3%). Microvascular invasion was observed in 16.6% of patients who met the Milan criteria and in over half of patients beyond the Up-to-seven criteria[124].

Recently, Mazzaferro *et al*[136] attempted to identify factors associated with HCC-related deaths of patients who underwent LT and to provide a predictive model of survival. The number of lesions and their size, as well as the levels of AFP, were significantly associated with HCC-specific deaths. To ensure an HCC-specific post-transplant survival rate of at least 70%, the authors suggested that the sum of the number and size of tumors (in centimeters) should not exceed 7 when the level of AFP was < 200 ng/mL, should not exceed 5 when the level of AFP was 200-400 ng/mL and should not exceed 4 when the level of AFP was 400-1000 ng/mL. Several studies confirmed the predictive value of pretransplant

AFP levels for HCC recurrence[22,137]. A similar role has also been demonstrated for PIVKA-II (5-fold increased risk for recurrence after transplantation)[138].

An approach independent of the size of the HCC and the number of lesions has been hypothesized through the "extended Toronto criteria"[137]. The eligibility for LT included tumors that do not have extrahepatic spread and/or macrovascular invasion and that have a low histological grade on preoperative biopsy and the patient enjoys a high performance status. The post-transplant survival rates in this case were shown to be independent of the patient's status within or beyond the Milan criteria (5-year overall survival of 69% and 78% for patients who met or did not meet the Milan criteria, respectively, $P = 0.3$) and therefore independent of the size of HCC and the number of lesions[139].

Furthermore, growing evidence suggests that an effective and sustained tumor downstaging with locoregional, surgical or systemic therapies from beyond to within the Milan criteria favorably impact overall and tumor-free survival[25,140,141]. In particular, the success rate of downstaging reported in the literature was higher than 40%[142]. Patients eligible for LT who met the Milan criteria after HCC downstaging showed 5-year tumor event-free and overall survival of 76.8% and 77.5%, respectively, compared to 18.3% ($P = 0.003$) and 31.2% ($P = 0.035$) of patients who underwent standard of care (non-transplantation therapies)[25]. Regardless of the Milan criteria, in patients with HCC eligible for LT, a pretransplantation loco-regional therapeutic approach (neoadjuvant, thermal ablation) reduced the risk of recurrence[4].

CONCLUSION

HCC is currently a potentially curable disease. However, recurrence rates still appear to be extraordinarily high. Although several factors are involved, the identification of predictive factors for both early and late HCC recurrence could optimize treatment strategies (LT, LR, thermal ablation) and surveillance. High-risk patients should be referred to treatments with the lowest recurrence rate (*e.g.*, LT [121]) and/or combination therapies, as well as intensive surveillance programs. The combination of multiple treatments or use of adjuvant or neoadjuvant therapeutic schemes (*e.g.*, immunotherapy) could reduce the recurrence rate and improve overall survival[143]. Research should therefore aim to validate these combined therapeutic strategies, particularly in patients with strong pre- and postoperative predictors of early or late recurrence.

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FOOTNOTES

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Country/Territory of origin: Italy

ORCID number: Riccardo Nevola 0000-0003-3320-3878; Ernesto Claar 0000-0002-0819-6023; Domenico Cozzolino 0000-0003-2009-5193; Ferdinando Carlo Sasso 0000-0002-9142-7848; Aldo Marrone 0000-0001-7329-4159; Luigi Elio Adinolfi 0000-0001-8453-4912; Luca Rinaldi 0000-0002-6541-3821.

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REVIEW

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Duc Long Tran, Palittiya Sintusek

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Duc Long Tran, Thailand and Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Bangkok, Thailand

Duc Long Tran, Faculty of Medicine, Can Tho University of Medicine and Pharmacy, Can Tho City 9000, Viet Nam

Palittiya Sintusek, Thai Pediatric Gastroenterology, Hepatology and Immunology Research Unit, Division of Gastroenterology, Department of Pediatrics, King Chulalongkorn Memorial Hospital and Thai Red Cross, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

Corresponding author: Palittiya Sintusek, MD, PhD, Doctor, Lecturer, Thai Pediatric Gastroenterology, Hepatology and Immunology Research Unit, Division of Gastroenterology, Department of Pediatrics, King Chulalongkorn Memorial Hospital and Thai Red Cross, Faculty of Medicine, Chulalongkorn University, 1873 Rama IV Road, Pathumwan Subdistrict, Bangkok 10330, Thailand. palittiya.s@chula.ac.th

Abstract

Functional constipation (FC) is considered the most common functional gastrointestinal disorder in children with a pooled global prevalence of 14.4% (95% confidence interval: 11.2-17.6) when diagnosed based on the Rome IV criteria. Its pathophysiological mechanisms are thought be multifactorial and complicated, resulting in difficult management. Currently, the most effective medication, when used in parallel with toilet training, is osmotic laxatives. Children's adherence to medication and parental concern regarding long-term laxative use are the main contributors to treatment failure. Recently, novel therapies with a high safety profile have been developed, such as probiotics, synbiotics, serotonin 5-hydroxytryptamine 4 receptor agonists, chloride channel activators, and herbal and transitional medicines; nonetheless, well-designed research to support the use of these therapies is needed. This review aims to focus on multiple aspects of FC in children, including global prevalence, pathogenesis, diagnostic criteria, tools, as well as conventional and novel treatment options, such as non-pharmacological management, including adequate fiber and fluid intake, physiotherapy, or neuromodulators. We also report that in very difficult cases, surgical intervention may be required.

Key Words: Constipation; Children; Laxative; Treatment; Toilet training; Herbal

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Core Tip: Functional constipation (FC) is a typical symptom of functional gastrointestinal disorders in children and its prevalence is high worldwide. Since the pathophysiology of FC in children is associated with stool withholding behavior, successful toilet training in combination with osmotic laxatives is crucial for the treatment childhood FC. Additionally, promising and innovative drugs can also aid in treatment success.

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INTRODUCTION

Functional constipation (FC) is considered a great disease burden in children that needs early screening and detection. The prognosis of FC is better in children with prompt and proper management. General physicians and pediatricians are usually the first person who take care these children hence understanding the pathophysiology of FC can lead to the proper management and satisfied outcome. In this chapter, the content will be covered all aspect the physician should know about FC for better patient care.

EPIDEMIOLOGY

Globally, up to 25% of visits to pediatric gastroenterologists and 3% of all general pediatric outpatient visits are due to FC[1]. It is difficult to determine the true prevalence of FC in children due to the heterogeneity of the studies in terms of target population sampling, diagnostic criteria, participant ethnicity and environment, method of data acquisition, and life style and psychological factors among others[2]. The primary reasons for the global diversity in prevalence among published studied may be due to the lack of agreement on diagnostic standards and cultural differences[3]. A systematic review and meta-analysis published by Koppen *et al*[4] reported that the worldwide prevalence of FC according to the Rome III criteria was 9.5% [95% confidence interval (CI): 7.5%-12.1%], with significantly more American and European children being affected than Asian ones. Additionally, geographical region, diet, and exposure to traumatic life events were linked to FC in children.

We determined that the pool global prevalence of FC in children was 14.4% (95%CI: 11.2-17.6) using the Rome IV criteria. According to continent, Africa had a highest prevalence of constipation (31.4%), followed by America (12.1%, 95%CI: 9.1%-15.1%), Europe (8.3%, 95%CI: 3.7%-12.9%), and Asia (6.2%, 95%CI: 1.3%-11%). Moreover, the factors significantly associated with FC from these studies are summarized in Figure 1 and Table 1.

NORMAL DEFECATION AND FC

Frequency of toileting habits in infants and children varies with age. To reduce parental worry and prevent needless testing and treatment, knowing the typical toilet routines for all age groups is important[5]. The frequency of stool passage per day gradually decreases from more than four times per day during the first week of life to three times per day at 4-6 wk of life and one to two times per day by the age of 4 years[6,7]. Healthy infants who are exclusively breastfed will have infrequent stool passage at 1-2 mo of life, with a mean duration of 6 days per stool passage (2-28 d per stool passage) without any abnormalities[8]. This condition will normalize at a mean age of 3.9 mo (range 1-7 mo)[9]. Hence, if the stool is soft, the infrequent stool passage in this age group requires neither intervention nor treatment. From the age of five, the majority of children pass stools daily or every other day without straining or withholding[3]. In early newborns, the average intestinal transit time is around 8.5 h, whereas intestinal transit times after puberty range from 30 to 48 h[5].

FC in adults had been first defined in 1999 according to the Rome II criteria and was mostly based on expert opinion. The diagnostic criteria of FC in children was subsequently established and integrated in Rome III criteria by Rome foundation in 2006. In 2016, the Rome III criteria had been replaced by the Rome IV criteria, with only minor changes being made as shown in Table 2[10]. Children who are not yet toilet trained do not need to include fecal incontinence (FI) and clogged toilet in the diagnostic criteria. In addition, the duration of symptoms in children had been changed from 2 mo in the previous Rome III criteria to 1 mo in the current Rome IV criteria[10,11] to promote early recognition and timely

Table 1 Prevalence of functional constipation in children according to Rome IV and factors associated with functional constipation

Ref.	Country	Population	Sample size	Age	Method of data collection	Prevalence of FC, *n*	Factors associated with FC
Huang *et al* [139], 2021	China	4 community hospitals in Jinhua and Shanghai	2604	0-4 yr	Guardian interview	92 (3.5%)	Vaginal delivery (OR = 0.01, 95%CI: 0.00-0.17), forceps delivery (OR: > 999, 95%CI: 154 to > 999)
Chew *et al*[140], 2021	Malaysia	A well child clinic, University Malaya medical center	534	1-12 mo	Guardian interview	6 (1.1%)	NA
Ibrahim *et al* [141], 2020	Egypt	Randomly schools in Cairo	1082	4-18 yr	NA	91 (8.4%)	NA
Khayat *et al* [142], 2021	Saudi Arabia	Public survey (random) from Western	317	3-18 yr	Questionnaire by Google form/links share on social apps	15 (4.7%)	Guardian characteristics, family income, gender, age, development, previous covid ì nfection ($P > 0.05$)
Benzamin *et al* [29], 2022	Bangladesh	Schools of Dhaka division	707	5-16 yr	Child interview and examination (face to face)	134 (19%)	Female ($P = 0.003$), age ($P = 0.001$), history of FC in siblings/parents ($P = 0.001$), fibers intake ($P = 0.002$), fluid intake ($P = 0.001$), electronic screen time ($P = 0.001$)
Siajunboriboon *et al*[143], 2022	Thailand	2 high schools	1700	14-18 yr	Child interview	138 (8.1%)	Guardian characteristics, BMI, history of allergic diseases ($P > 0.05$)
Chia *et al*[144], 2022	Vietnam	A government hospital and a government kindergarten	1511	0-48 mo	Guardian interview and examination (face to face)	46 (3%)	Male (OR = 3.6, 95%CI: 1.5-8.5), bottle feeding (OR = 18.5, 95%CI: 1.5-219.4), low income (OR = 5.8, 95%CI: 1.7-19.3)
Asia			**8455**			**522 (6.2%)**	
Zwiener *et al* [145], 2017	United States	Online survey	1075	4-18 yr	Guardian interview	144 (13.4%)	NA
Saps *et al*[146], 2018	Colombia	12 schools in 6 cities	3567	8-18 yr	Child interview	382 (10.7%)	NA
Robin *et al*[147], 2018	United States	Online survey panels by CINT, United States	1255	0-18 yr	Guardian interview	186 (14.8%)	NA
Játiva-Mariño E *et al*[148], 2019	Ecuador	1 public and 1 private school	951	8-15 yr	Child interview	137 (14.4%)	NA
Saps *et al*[149], 2020	Colombia	6 outpatient clinics	1334	1-48 mo	Guardian interview (face to face)	281 (15.1%)	NA
Velasco-Benitez *et al*[150], 2020	Colombia	4 public schools	1497	10-18 yr	Guardians interview	194 (13%)	NA
Baaleman *et al* [151], 2021	Colombia	A public school	118	11-18 yr	Child interview	16 (13.6%)	NA
Velasco-Benítez *et al*[152], 2021	Colombia	5 to 8 grade students in Cali	465	10-18 yr	Children interview	52 (28.7%)	NA
Dos Santos *et al* [27], 2021	Brazil	Public parks and school areas	799	5-14 yr	Guardian interview	163 (20.4%)	Sex, type of school ($P > 0.05$)
de Morais *et al* [153], 2022	Brazil	Pediatric private clinics in 5 regions	4560	0-12 mo	Guardian interview	341 (7.6%)	Age 162-248 d (OR = 1.41, 95%CI: 1.01-1.95), prematurity (OR = 1.44, 95%CI: 1.02-2.02)
America			**15621**			**1896 (12.1%)**	
Russo *et al*[21], 2019	Italy	General clinics	214	1 mo-17 yr	Guardian/child interview(face to face)	39 (18.2%)	NA
Vladimir *et al* [154], 2019	Russia	University clinic	300	0-48 mo	Guardian interview	45 (15%)	NA
Steutel *et al* [155], 2020	Belgium, Italy, Netherland	General pediatrics hospital (Belgium, Italy) and well-baby clinic (the Netherlands)	2751	0-48 mo	Guardian interview and examination (face to face)	151 (5.4%)	NA
Campeotto *et al*	France	Private out-patient	1570	0-12	Guardian interview	141 (9%)	NA

[156], 2020		pediatricians and general practitioner		mo	and examination (face to face)		
Alonso-Bermejo *et al*[157], 2022	Spain	A pediatric gastroenterology clinic	574	0-16 yr	Guardian/child interview and examination (face to face)	41 (7.1%)	NA
Beser *et al*[158], 2021	Turkey	9 tertiary Hospital	2383	1-12 mo	Child interview and examination (face to face)	112 (4.7%)	NA
Strisciuglio *et al* [159], 2022	6 Mediterranean countries[1]	Nursery schools, primary schools and secondary schools, randomly	4353	4-18 yr	Guardian interview	475 (10.9%)	NA
Europe			**12145**			**1004 (8.3%)**	
Bellaiche *et al* [160], 2020	10 countries in Africa[2]	Children with gastrointestinal symptoms	10458	0-12 mo	Guardians interview and examination (face to face)	3283 (31.4%)	NA
Africa			**10458**			**3283 (31.4%)**	
All over the world			**46679**			**6704 (14.4%)**	

[1]6 Mediterranean countries: Croatia, Greece, Israel, Italy, Macedonia, Serbia.
[2]10 countries: Algeria, Cameroon, Congo, Gabon, Madagascar, Morocco, Mauritius, Ivory Coast, Senegal, Tunisia.
CINT: A global software in digital insight and research technology; FC: Functional constipation; CI: Confidence interval; OR: Odds ratio; apps: Applications; NA: Not available; BMI: Body mass index.

Table 2 Rome IV criteria for pediatric functional constipation[6,7,9]

Child's age	Diagnostic criteria
< 4 years old	Two or more criteria for at least 1 mo[1]: (1) Two or fewer defecations per week; (2) History of excessive stool retention; (3) History of painful or hard bowel movements; (4) History of large diameter stools; (5) Presence of a large fecal mass in the rectum; (6) At least one episode of fecal incontinence per week after the acquisition of toileting skills; and (7) History of large-diameter stools that may obstruct the toilet in toilet trained children
≥ 4 years old	Two or more symptoms for at least 1 mo in children at least 4 yr[2]: (1) Two or fewer defecations per week; (2) At least one episode of fecal incontinence per week; (3) History of retentive posturing or excessive stool retention; (4) History of painful or hard bowel movements; (5) Presence of a large fecal mass in the rectum; (6) History of large-diameter stool that may obstruct the toilet; and (7) Additional criteria: Without fulfilling irritable bowel syndrome criteria

[1]After appropriate evaluation, the symptoms cannot be fully explained by another medical condition.
[2]After appropriate evaluation, the symptoms cannot be fully explained by another medical condition.

treatment. Although there are some changes of the diagnostic criteria in Rome IV, the prevalence of FC in children was similar using either the Rome IV or Rome III criteria.

FI in children is defined as the involuntary passage of stool into the underwear either as unintentional seepage of small amounts of liquid stools (generally referred to as "soiling" or "leakage") in a child older than 4 years of age or in a toilet-trained child[12,13]. Irrespective of the amount of stool, this is one of the most unpleasant and embarrassing things for a growing child apart from being an upsetting and mentally distressing issue that has a negative impact on children's quality of life. FI was divided into 2 types; retentive and nonretentive FI. It is critical to distinguish between retentive and nonretentive FI given their different etiologies and approaches to treatment[14]. Hospital and community studies have shown retentive FI occurs in constipated children with fecal impaction[1,15,16], whereas the nonretentive type could be found in children with psychological problems[3].

PHYSIOLOGY OF DEFECATION AND PATHOGENESIS

The act of defecation is a process related to the pelvic floor muscles, anal sphincter complex, enteric nervous system, and central nervous system (Figure 2). Normally, children over 18 mo old can initially control defecation through this complicated process, with nearly all of them succeeding in controlling defecation by the age of 4.

The etiology of constipation can be classified into functional and organic causes, which account for 90% and 10% of the cases, respectively[12]. Regarding FC, the pathophysiological mechanism might be multifactorial, including stool withholding behavior, anorectal dysfunctions, diet, physical activity,

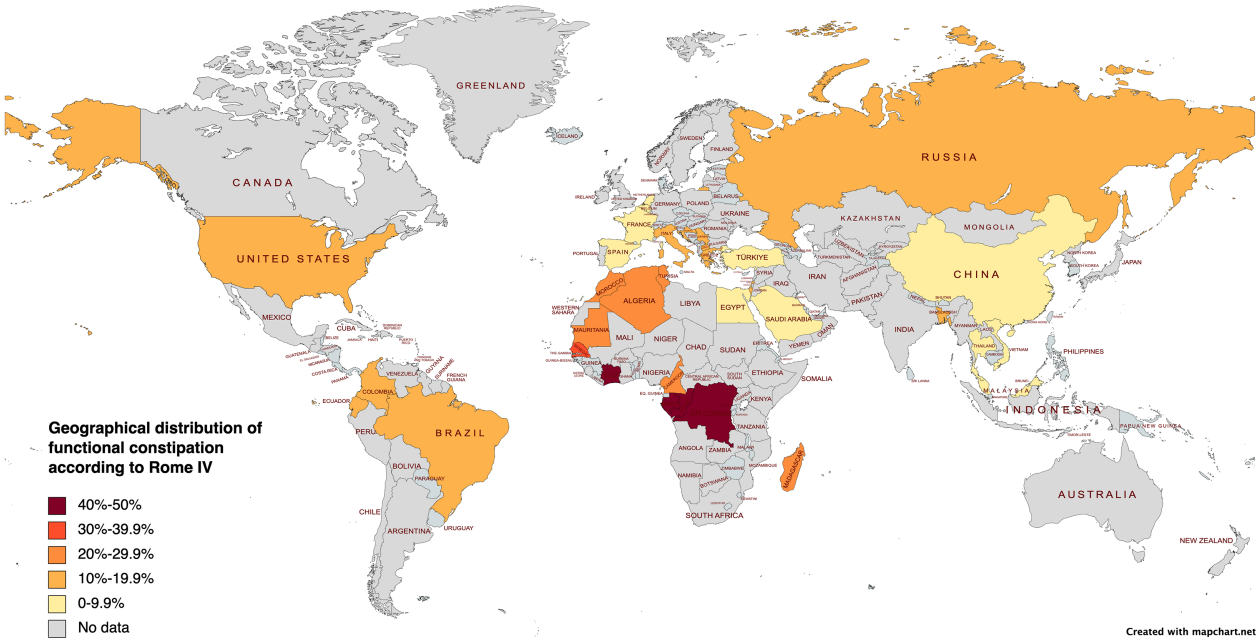


Figure 1 Geographical distribution of functional constipation in children worldwide, according to Rome IV.

genetic predisposition, and psychological issues. Stool withholding behavior is the main pathophysiological mechanism especially in toddlers and young children. Faulty toilet training, painful defecation from hard stool and frequent rectal enema contribute to fear and bad experiences related to defecation, which can cause purposeful or subconscious stool withholding behavior. Instead of relaxing the pelvic floor muscle when feeling the urge to defecate, children will defecate in the standing position and contract the pelvic floor and gluteal muscles, a phenomenon called "retentive posture or defecation in standing position". This behavior promotes the retention of stool in rectum and causes the stool to become lumpier and harder, making it quite difficult to evacuate, due to water absorption by rectal mucosa. This phenomenon leads to a vicious cycle of difficult defecation. Once large stools are retained in the rectum, the rectal wall stretches and develops into a megarectum[17] with decreased sensation to defecate[12]. Moreover, liquid stool can penetrate the hard stool and leak out of the anus, causing fecal soiling (Figure 3). According to pathophysiology, withholding behavior, palpable fecal mass on abdominal examination, and fecal soiling were reported in 37%-91%, 33%-68%, and 33%-77% of in children with FC, respectively[18-21]. Hence, two of the three characteristics were integrated into the Rome IV criteria.

CLINICAL MANIFESTATIONS OF AND EXAMINATIONS FOR FUNCTIONAL AND ORGANIC CONSTIPATION

FC is diagnosed based on symptoms detailed in the Rome IV criteria. However, some examinations may help pediatricians in cases with uncertain symptoms and signs[22]. Moreover, in cases that are difficult to treat or have alarm features (Table 3), examinations to exclude organic cause are necessary. Here we will review the clinical manifestations and examinations that can helpful general pediatricians and specialists diagnose functional and organic constipation (Table 4).

Patient history

When obtaining the medical history of children, it is important to inquire about when the child had their first bowel movement after delivery. Normally, during the first 24 h of life, more than 90% of term newborns pass meconium[23,24]. This period may be longer in preterm infants due to the delayed maturation of the intestinal motor function[25]. If the passage of meconium is delayed after birth, worrisome diseases, such as Hirschsprung's disease (HD) and cystic fibrosis, should be excluded[6,26]. Age of onset, frequency, consistency and size of the stool, painful or difficult defecation, and presence of blood coating the stool are all crucial details to note when recording a patient's history. In addition, frequent clogging of the toilet might reflect a large fecal mass in rectum. Anal fissures should be examined in children with a history of difficult defecation (Figure 4A) and those with blood coating the stool (Figure 4B) or the toilet paper. It is necessary to gather information regarding incontinence or soiling during the day and night. FI can be mistaken for diarrhea by their guardians[1,6] (Figure 4C).

Table 3 Alarm signs and symptoms of constipation[37]

Alarm signs	Symptoms of constipation
History	Constipation starting in neonatal period, delay pass meconium (> 48 h of life), family history of Hirschsprung's disease
Stool characteristics	Ribbon stools, blood in the stools in the absence of anal fissures
Gastrointestinal features	Bilious vomiting, severe abdominal distension
Back	Sacral dimple, tuft of hair on spine, gluteal cleft deviation
Anus	Perianal fistula, abnormal position of anus, anal scar, absent anal/cremasteric reflex
Neurological features	Decreased lower extremity strength/tone/reflex
Others	Abnormal thyroid gland, fever, faltering of growth

Table 4 Organic causes of constipation[1]

Organic causes	
Abnormalities of colon and rectum	Anal or colonic stenosis. Imperforate anus. Anteriorly displaced or ectopic anus. Cloacal malformations. Chronic intestinal pseudo-obstruction
Systemic disorders	Hypothyroidism. Hypercalcemia. Hypocalcemia. Diabetes mellitus. Panhypopituitarism. Cerebral palsy. Myotonia congenita. Scleroderma. Amyloidosis. Mixed connective tissue disease. Myotonic dystrophy. Progressive systemic sclerosis
Others	Cystic fibrosis. Celiac disease. Heavy metal ingestion (lead, mercury)
Spinal cord abnormalities	Meningomyelocele. Spinal cord tumor. Sacral agenesis. Tethered cord
Neuropathic intestinal disorders	Hirschsprung's disease. Intestinal neuronal dysplasia. Chagas disease. Abnormal muscle of abdomen. Prune belly syndrome. Gastroschisis
Drugs	Opiates. Anticholinergics. Antacids. Antihypertensives. Antimotility agents. Cholestyramine. Psychotropics. Diuretics

Evidence of fecal impaction in children suspected of FI is crucial, and physicians could obtain this information through abdominal palpation of a fecal mass, digital rectal examination, or rarely through plain abdominal radiography in noncooperative children. Importantly, physicians must define how a child defecates. Withholding behaviors are considered the main pathogenesis of FC and have been defined by guardians as defecation in the "standing position". This can also be described as stiffening up, buttock clenching, walking on tip toes, crossing one leg over the other, bracing against furniture, being in the all-fours position or curling up in a ball, and sitting with legs straight out (Figure 4D)[27,28]. Withholding behaviors also help clinicians determine that the constipation should be of functional etiology without any organic problems. Other factors, such as significant life events like a family member's death, the birth of a sibling, difficulties in school, sexual abuse, and others, can contribute to retentive behaviors and FC and should be evaluated in detail[1,6].

Although the abdominal pain caused by FC is typically nonspecific and poorly localized, constipation was the cause of acute abdominal pain in 50% of children who presented for a primary care visit and should be considered in this context[1]. Physicians should identify alarm features (Table 4) and other signs and symptoms, including appetite loss, fever, nausea, vomiting, reduced weight gain, issues with neuromuscular development, and behavioral or psychological problems[6,26,29]. Poddar *et al*[18] reported that children with symptoms such as delayed passage of meconium, growth failure, lack of retentive posturing, and absence of fecal impaction may likely have organic FC. Furthermore, urinary tract infections have been reported in a significant number of children suffering from constipation and FI[30,31]. Dietary and constipation treatment history should be investigated to predict the long-term outcomes of FC in affected children[1,6,32].

Physical examination

Assessing children's physical development through weight and height measurements should be the first step[6]. Abdominal examination should obtain information on the rectal fecal mass, particularly its height above the pelvic brim, through bimanual palpation on either side of the rectus sheath[7]. With careful abdominal and digital rectal examination, fecal masses can be detected in 30%-75% of children with FC[1,18]. The perineum should be examined given that it can reveal important details regarding the anal position, evidence of FI, skin irritation, eczema, fissures, and signs of possible sexual abuse[6].

Measuring the anogenital index (Figure 5) is important given that it might be considered a factor associated with FC. The anogenital index can be calculated using the formula presented below[33-36]. The normal anogenital index in males and females is 0.54 ± 0.03 and 0.40 ± 0.04, respectively[35].

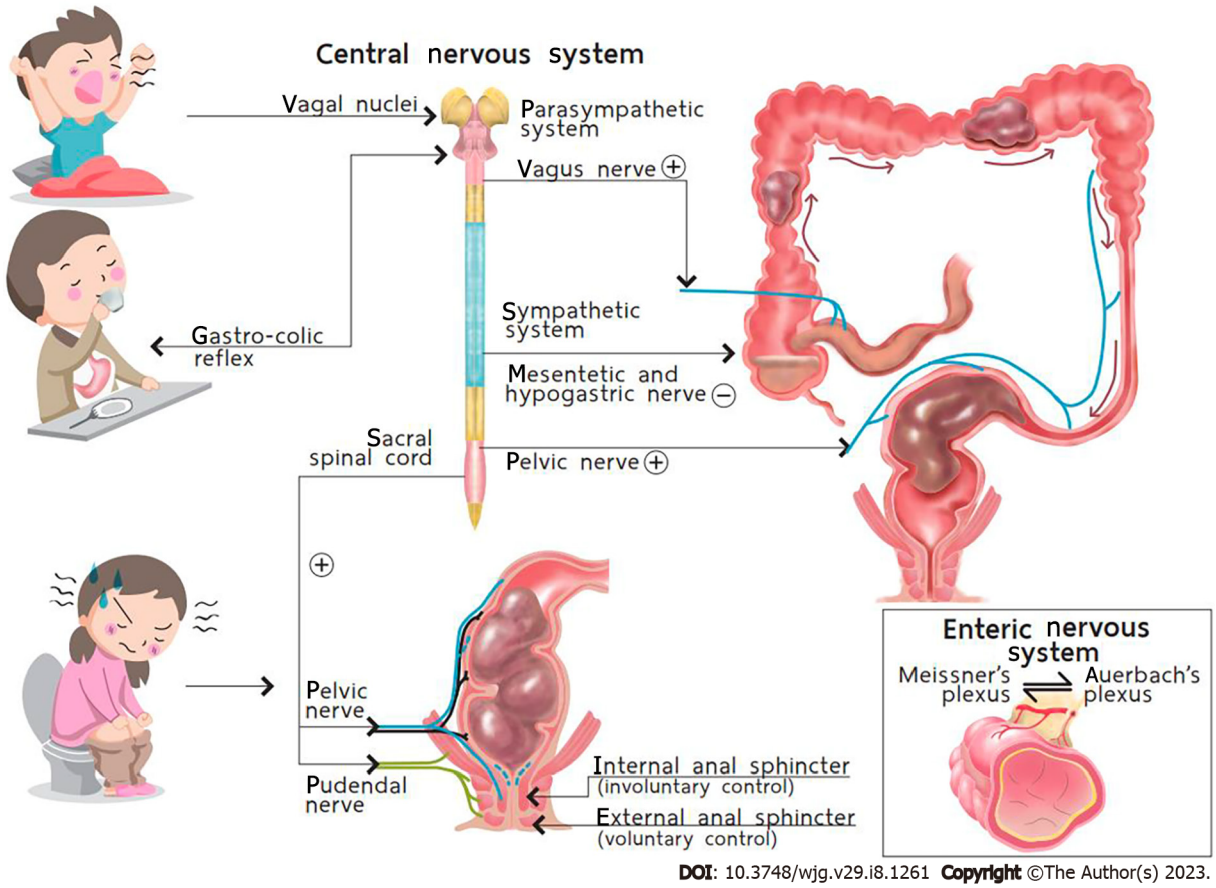


Figure 2 Normal defecation dynamics. Citation: Palittiya Sintusek. Pediatric lower gastrointestinal motility disorders. In: Werawatganon D, editor. Practical Neurogastroenterology and motility: Basic, testing, and treatment. Bangkok: Printable Inc, 2021: 417. Copyright ©The Authors 2021. Published by Thai Neurogastroenterology and Motility Society. The authors have obtained the permission for figure using from Thai Neurogastroenterology and Motility Society (Supplementary material).

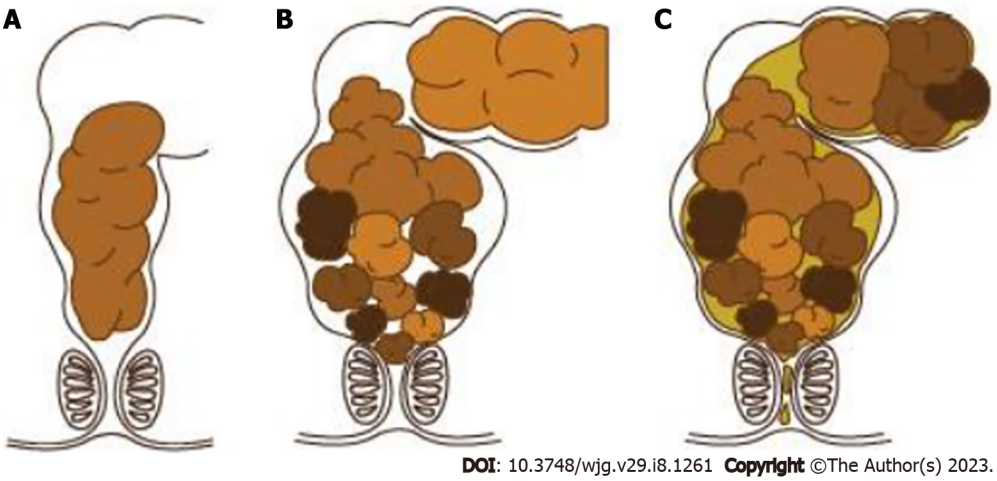


Figure 3 Accumulation of fecal mass in the rectum causes rectal wall distension and fecal soiling. A: Normal stool; B: Massive stool are retained in the rectum from withholding behavior; C: Liquid stool can leak out the anus or fecal soiling. A-C: Citation: Palittiya Sintusek. Chronic constipation. In: Supapitiporn K, editor. Pediatric practice: simple&applicable. Bangkok: Chulalongkorn University, 2021: 96. Copyright ©The Authors 2017. Published by Department of Pediatrics, Chulalongkorn University. The authors have obtained the permission for figure using from Department of Pediatrics, Chulalongkorn University (Supplementary material).

Anogenital index = [vagino/scroto to anal distance (cm) ÷ vagino/scroto to coccygeal distance (cm)] [35].

When the child's history suggests the presence of FC, a digital rectal examination may not be necessary[7,37]. Digital rectal examination should be conducted when children present with red flags, a



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Figure 4 Clinical manifestations in constipated children. A: Anal fissure usually at the 6 o'clock or posterior parts; B: Blood coated, hard, and lumpy stool; C: Rectal abrasion from fecal incontinence that was misdiagnosed as chronic diarrhea; D: Retentive posture. A-D: Citation: Palittiya Sintusek. Chronic constipation. In: Supapitiporn K, editor. Pediatric practice: simple&applicable. Bangkok: Chulalongkorn University, 2021: 96. Copyright ©The Authors 2017. Published by Department of Pediatrics, Chulalongkorn University. The authors have obtained the permission for figure using from Department of Pediatrics, Chulalongkorn University (Supplementary material).

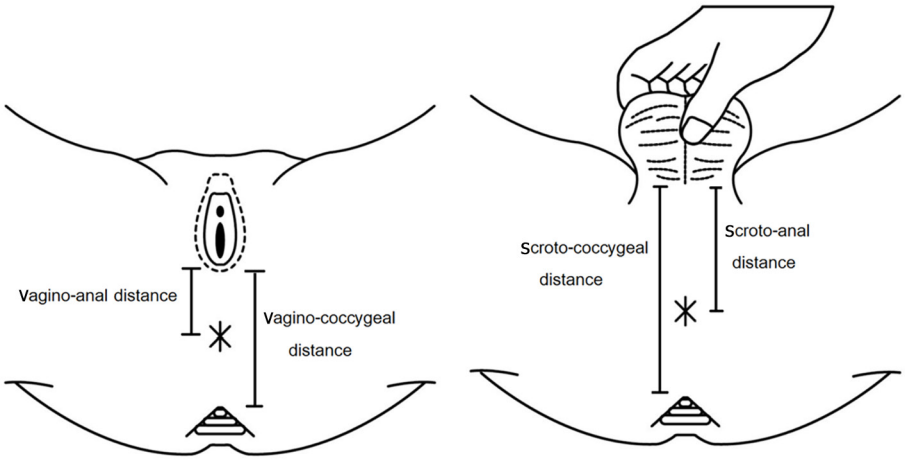


Figure 5 Landmarks for the measurement of the anogenital index. Citation: Palittiya Sintusek. Chronic constipation. In: Supapitiporn K, editor. Pediatric practice: simple&applicable. Bangkok: Chulalongkorn University, 2021: 96. Copyright ©The Authors 2017. Published by Department of Pediatrics, Chulalongkorn University. The authors have obtained the permission for figure using from Department of Pediatrics, Chulalongkorn University (Supplementary material).

history of delayed meconium passage after birth, intractable constipation, an uncertain diagnosis according to the Rome IV criteria, suspicion of an anatomic problem, and assessment of fecal impaction after disimpaction. Although neurological disease causing organic constipation is very rare, dedicated neurological examination still has merit (Figure 6).

Laboratory examinations

It is necessary to emphasize that FC is a clinical diagnosis based on a detailed medical history and physical examination. The goal of laboratory testing is to determine the presence of a rare organic



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Figure 6 Sacral dimple and spinal dysraphism on spinal radiography (arrow) in a 5-year-old boy with intractable constipation. A: Sacral dimple; B: Spinal dysraphism.

etiology in children with constipation showing alarm features[37] (Table 3) or confirm the diagnosis of FC in complicated or unclear cases. Hence, further diagnostic interventions are sometimes warranted. Investigations that might be useful for determining organic causes of constipation are described below.

Laboratory testing: Thyroid function and serum calcium tests can be used in children with intractable constipation or chronic constipation who are quite difficult to treat. In countries with a high prevalence of celiac disease and cystic fibrosis, specific tests for these diseases might be considered. Although the prevalence of food allergies in children presenting to tertiary clinics with chronic constipation who are unresponsive to traditional treatment vary from 28% to 78%[38], conflicting data support the use of allergy testing to identify cow's milk protein allergy (CMPA) in constipated children[39-41]. Therefore, according to the recommendations of the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) and North American Society of Pediatric Gastroenterology, Hepatology and Nutrition (NAPSGHAN), routine allergy testing is not recommended in constipated children suspected for CMPA[37]. Oral food avoidance and rechallenge is the gold standard for diagnosing CMPA that manifests with intractable constipation.

Abdominal radiography: Abdominal radiography can be help pediatricians determine the presence fecal masses in some cases where physical examination is limited (Figure 7), such as obese children, patient refusal, or noncooperation, or exclude some causes of acute abdominal pain.

According to systematic reviews, abdominal radiography can identify constipation with a sensitivity and specificity of 60%-80% and 43%-99%, respectively[42,43]. There was inconsistent evidence to support the diagnostic relationship between constipation symptoms and fecal loading in abdominal radiographs from children; hence, the results should be interpreted with caution. In line with this, three scoring systems have been developed, namely the Barr score[44], Leech score[45], and Blethyn score [46]. However, further validation is needed before they can be widely used in clinical practice.

Abdominal ultrasonography: Abdominal ultrasonography can assess stool retention and estimate the size of the rectum and colon based on the supposition that fecal retention is one of the primary characteristics of constipation in both children and adult[6,47]. Given that ultrasound scanning is noninvasive and radiation-free, it is usually used for assessment in primary and secondary clinical care[1,6]. Rectal diameter measurements were correlated with the results of digital rectal examination and therefore seems to accurately assess fecal impaction[43]. Evidence suggests that digital rectal examination might be replaced by ultrasound scanning given that the latter is less unpleasant[48]. Even though there was a good correlation of transverse rectal diameter with FI and long-term constipation[49], the transverse diameter cannot be used to predict fecal impaction or constipation[37]. Furthermore, the results are largely operator dependent, and patient cooperation is also needed.

Radiopaque marker for colonic transit study: Based on the distribution of markers throughout the colon, the radiopaque marker (ROM) for colonic transit time (CTT) study is one method for distinguishing between different types of colonic function, including normal colonic transit, slow-transit constipation, and obstruction of the rectal outlet. Given its accessibility and strong concordance with scintigraphic methods, the ROM for CTT study has become the most popular method for determining both total and segmental CTT[50]. The sensitivity and specificity of the ROM for CTT study were 71% (95%CI: 57%-83%) and 95% (95%CI: 82%-99%), respectively[51]. The mean transit time in healthy persons has been reported to range from 15.6 to 37.7 h, with a review by Southwell *et al*[52] revealing that the normal CTT was < 32 h (upper 95th centile: 54 h).

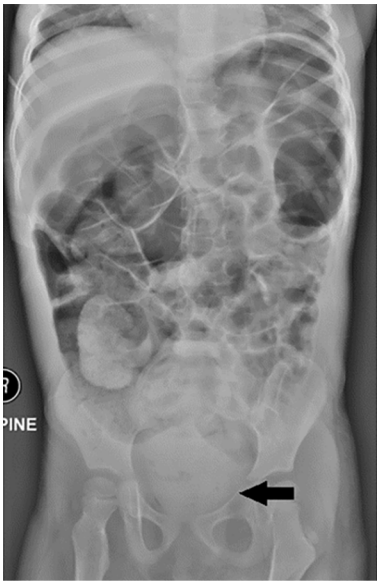


Figure 7 Abdominal radiography of a 3-year-old girl with a chief complaint of chronic diarrhea for 4 mo shows fecal impaction in the rectum. Citation: Palittiya Sintusek. Chronic constipation. In: Supapitiporn K, editor. Pediatric practice: simple&applicable. Bangkok: Chulalongkorn University, 2021: 96. Copyright ©The Authors 2017. Published by Department of Pediatrics, Chulalongkorn University. The authors have obtained the permission for figure using from Department of Pediatrics, Chulalongkorn University (Supplementary material).

The ESPGHAN and NAPSGHAN recommend that these tests only used to distinguish FC from functional non-retentive fecal incontinence or, where the diagnosis is unclear, provide clarity and allow the selection of alternative diagnostic procedures due to the widespread use of the Rome criteria for the diagnosis of FC and the potential risks caused from repeated radiation exposure from abdominal radiography[37,50].

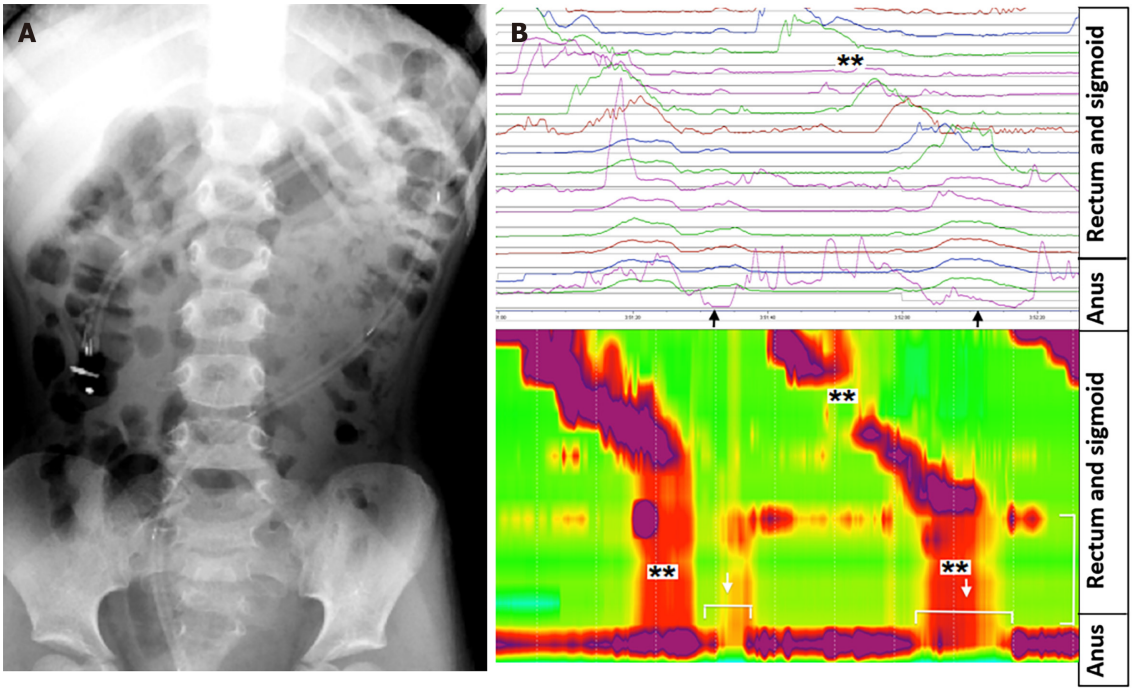
Colonic manometry: To determine the neuromuscular function of the colon in children with intractable constipation, colonic manometry is regarded as the gold standard[50]. Evidence suggests that colonic manometry is most useful in providing subsequent guidance for further therapy, including pharmacological and surgical management, in intractable constipation. According to a review by the ESPGHAN motility group, high amplitude propagating contractions are the most easily recognizable and reliable motor pattern (Figure 8). They are initiated usually in the proximal colon and expected to stop at the recto-sigmoid junction. However, colonic manometry can be difficult for children given its invasive nature and necessity for general anesthesia. Moreover, age can be a potential limiting factor depending on the size of the catheter and endoscope. Additionally, only a few specialist centers globally offer this test.

Wireless motility capsule: The wireless motility capsule (WMC) is a novel, nonradioactive, and minimally invasive tool for the assessment of colonic motor function. Several investigations have reported on the safety and tolerability of WMC. In recent years, an increasing number of studies have used WMC to diagnose children with functional gastrointestinal diseases (FGIDs)[53,54]. This test could provide information on gastrointestinal motility that is similar to information obtained *via* nuclear medicine gastric emptying time and/or ROM. Moreover, WMC can provide additional information on regional and entire-gut transit[53], which can not only add to our knowledge of colon physiology but also be used as a parameter to tailor treatment[54]. Considering its safety and low invasiveness, the ESPGHAN motility working group recommended that more research be done to assess the effectiveness of the WMC in predicting outcomes among children with intractable constipation[50].

Magnetic resonance imaging: Cine magnetic resonance imaging (cMRI) is a noninvasive tool that uses a high-resolution spatiotemporal approach to facilitate dynamic MRI, which would allow the observation of the gut lumen diameter. The assessment of stomach accommodation and emptying, terminal ileum motility, and the small bowel using cMRI has been documented in the literature[43].

In both adults and children, cMRI can be used to assess colonic motility for various gastrointestinal disorders[55]. However, data on the application of this method in children with intractable FC are currently scarce.

Vriesman *et al*[56] published the first pediatric study comparing the identification of colonic motility patterns on cMRI with that on colonic manometry, proving potential evidence regarding the feasibility of the technique. cMRI has the advantage of being noninvasive, precluding the need for general anesthesia; however, given that this is still a research-based modality, additional studies are required to establish objective and systematic measurements.



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Figure 8 High amplitude propagating contractions. A: Proper position of the colonic catheter; B: graphical demonstration of abnormal high amplitude propagating contractions (HAPCs) with absent HAPCs at the sigmoid region (**) but preserved colo-anal reflex (arrow). A and B: Citation: Palittiya Sintusek. Chronic constipation. In: Supapitiporn K, editor. Pediatric practice: simple&applicable. Bangkok: Chulalongkorn University, 2021: 96. Copyright ©The Authors 2017. Published by Department of Pediatrics, Chulalongkorn University. The authors have obtained the permission for figure using from Department of Pediatrics, Chulalongkorn University (Supplementary material).

Barium enema and others: A barium enema is used to coat the lining of the colon and rectum in order to create clearer images of the colon (Figure 9A). A contrast enema is often used in the diagnostic workup of HD, in which a transition zone between the aganglionic and ganglionic bowel may be observed in histopathology study[6] (Figure 9B). A 24-h delayed barium enema film could offer comprehensive data on colon transit function in young children, especially those under 4 years old who often cannot undergo CTT study[57]. Moreover, delayed retention of contrast at 48 h provides the strongest negative predictive value to exclude HD. Nonetheless, a limitation of barium enema is that it cannot be used to diagnose ultrashort HD; instead, anorectal manometry is required wherein absence of the anorectal inhibitory reflex is pathognomonic for HD (Figure 9C).

TREATMENT OPTIONS

The goals of treatment include establishing regular defecation (ideally once a day, passing soft stools and without difficulties) and preventing relapses[1,6,58]. Oral laxatives and structured toilet training are the main tools of a successful treatment. FC in children is typically managed across four important phases: (1) Education; (2) Fecal disimpaction; (3) Preventing fecal reaccumulation; and (4) Follow-up.

Education

Education is an important initial step of treatment[59] given its association with adherence and the successful of management of constipated children. In fact, studies by Steiner *et al*[60] and Koppen *et al*[61] reported that 38% and 37% of patients adhered to therapy, respectively. Inconvenience, dissatisfaction with treatment, and emotional impact of symptoms were linked to low adherence, all of which require attention[61].

Therefore, education should include an explanation of the physiological dynamics of defecation; associated factors of constipation; and the related shame, embarrassment, and social issues to the guardians and their children. In particular, the doctor needs to make it clear to the family that withholding behavior is crucial to the pathophysiology of FC. Furthermore, physicians should create a thorough plan to eliminate the frustration of guardians and children and increase cooperation required for prolonged treatment. Moreover, the timing of a successful treatment is frequently unpredictable, and guardians must understand that there is no quick fix for this problem. Recovery is only feasible with a sufficient, frequent, and prolonged care[1,6,59]. In some complex cases, such as in children with

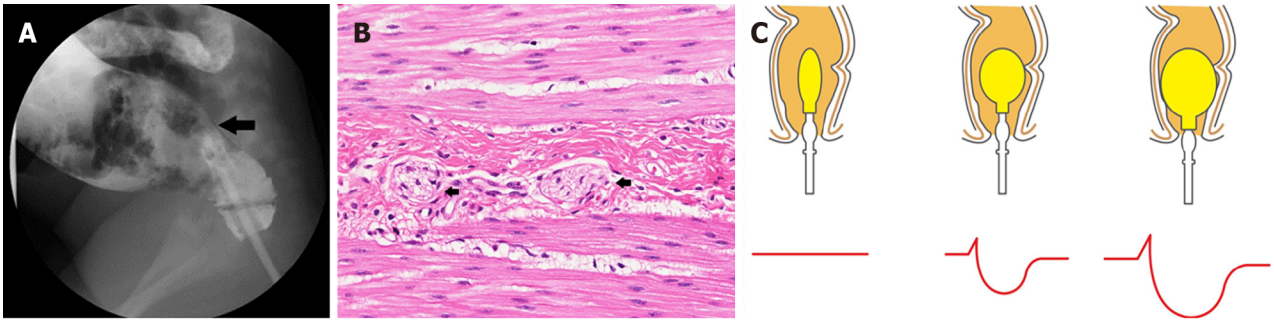


Figure 9 Investigations for organic constipation. A: Transition zone after barium enema in a child diagnosed with Hirschsprung's disease; B: Histopathology revealing no ganglion cells in the nerve plexuses (myenteric plexus) of the muscular propria layer. Arrow shows thick nerve trunk but without ganglion cells; C: Anorectal manometry showing rectoanal inhibitory reflex. A-C: Citation: Palittiya Sintusek. Pediatric lower gastrointestinal motility disorders. In: Werawatganon D, editor. Practical Neurogastroenterology and motility: Basic, testing, and treatment. Bangkok: Printable Inc, 2021: 417. Copyright ©The Authors 2021. Published by Thai Neurogastroenterology and Motility Society. The authors have obtained the permission for figure using from Thai Neurogastroenterology and Motility Society (Supplementary material).

intractable constipation or in those suffering from other comorbidities such as urinary problems, it is necessary to involve a multidisciplinary team that includes pediatric specialist nurses, pediatric research nurses, psychiatrists, urotherapists, and urologists for long-term follow-ups. Hence, such children require customized care.

Fecal disimpaction

Fecal disimpaction is crucial before the start of maintenance therapy in order to maximize the success of treatment. If fecal impaction is not eliminated beforehand, maintenance therapy can lead to worsening FI[1,6,59]. Oral drugs, rectal enema, or a combination of both can effectively treat fecal impaction. Two randomized controlled trials (RCTs) showed that polyethylene glycol (PEG) and enemas are equally effective for fecal disimpaction[62,63]. The use of 1-1.5 g/kg/d of PEG with or without electrolytes orally for 3-6 d is recommended as the first-line treatment for constipated children with fecal impaction [37]. However, a RCT study reported that both lactulose and PEG treatment successfully promoted disimpaction and were safe and well tolerated, although PEG achieved disimpaction significantly faster at day 2 (P = 0.001)[64]. Lactulose may be a useful PEG substitution for treating fecal impaction, particularly in areas wherein the availability of PEG is limited.

The oral route is typically less intrusive, better tolerated, and provides children with a better sense of control than rectal enema; however, successful disimpaction can take a few days, with compliance also being an issue. In contrast, the rectal approach (enema) is faster (effect occurs within minutes) but more invasive and traumatic. It may be useful for removing fecal impaction in patients with severe abdominal pain or large fecal mass on abdominal examination. Common side effects of enema include anorectal discomfort and abdominal pain; hence, it should be avoided in scared and resistant children to avoid anal fissures[58]. Recently, a study on the efficacy of olive oil enemas for childhood constipation showed that 79.6% and 66.7% of FC cases in the olive oil and lubricant groups were effectively treated for fecal impaction, respectively[65] (Table 5).

Maintenance therapy

Following disimpaction, maintenance therapy should be started immediately. The goals of maintenance therapy are to produce soft and painless stools, avoid stool reimpaction, and stop the reemergence of stool withholding behavior. This can be accomplished by combining pharmacological and nonpharmacological interventions.

Pharmacological management

Osmotic laxatives: Osmotic laxatives, including PEG, lactulose, and milk of magnesium hydroxide (MOM), are a type of osmotically active ions or molecules that are rarely absorbed in the small intestine. As such, they stimulate water retention in the colon, consequently softening stools.

PEG has been demonstrated to be more effective at increasing bowel movement frequency than lactulose, making it the first option for maintenance therapy in constipated children[66-68] or MOM [69]. However, limited evidence of its the utilization and safety has been available in infants, especially for long-term usage[66,70]. Moreover, data from a 10-year survey revealed that 645 children using PEG 3350 between the ages of 0-21 reported 1564 adverse symptoms. Among these adverse symptoms, 58.75% were neurological or neuropsychiatric, such as anxiety, anger, abnormal behaviors, and others [71]. However, the data source from this survey had significant limitations, including sampling bias, lack of verification, inability to seek clarifications, and lack of follow-up data. Comparing the efficacy of lactulose and MOM, one study found a significant difference in the frequency of stool passage per week,

Table 5 Laxatives used for functional constipation in children[1,6,37,58]

Agent	Child's age	Dosage	Side effects
Osmotic laxatives			
PEG	Any age	0.4-0.8g/kg per day for maintenance; 1-1.5g/kg per day for fecal disimpaction	Diarrhea, bloating, flatulence, nausea, vomiting, abdominal cramps
Lactulose (70% solution)	Any age	1 mL/kg once or twice daily (max 120 mL per day)	Bloating, flatulence, abdominal cramps, fecal, incontinence
Sorbitol (70% solution)	1-11 yr	1 mL/kg once or twice daily (max 30 mL per day)	Bloating, abdominal cramps
	> 12 yr	15-30 mL once or twice daily	
Milk of magnesium	> 2 yr	1-3 mL/kg per day once or twice daily	Abdominal pain, fecal incontinence, hypermagnesaemia, hypocalcaemia, hypophosphataemia (with excess use in children with renal disease)
Stimulant laxatives			
Senna (antraquinone)	> 2 yr	7.5-15 mg/kg per day once daily	Abdominal cramps, idiosyncratic hepatitis, melanosis coli in prolong used, nephropathy, neuropathy, hypertrophic osteoarthropathy
Bisacodyl	> 2 yr	5-10 mg per day once daily	Diarrhoea, abdominal cramps
Sodium picosulphate	4-5 yr	3 mg per day	Nausea, vomiting, bloating, abdominal cramps, diarrhea, headache, taste impairment
	> 6 yr	4-6 mg per day	
Glycerine suppository	< 1 yr	Half for pediatric suppository once daily	Rectal irritation, bloating, abdominal cramps, diarrhea
Rectal laxatives/enemas			
Sodium phosphate	> 1 yr	2.5 mg/kg	Rectal discomfort, diarrhea, abdominal cramps, electrolyte imbalance
Bisacodyl	2-12 yr	5 mg/dose once daily	Rectal discomfort, diarrhea, abdominal cramps, hypokalemia
	> 12 yr	5-10 mg/dose once daily	
Saline enema	Neonate	< 1 kg: 5 mL, > 1 kg: 10 mL	Rectal discomfort, bloating
	> 1 yr	6 mL/kg once or twice daily	
Lubricant			
Mineral oil	> 1 yr	1-2 mL/kg daily (max 90 mL per day)	Rectal discomfort, lipoid pneumonitis

PEG: Polyethylene glycol.

favoring MOM over lactulose (MD: 1.51, 95%CI: -2.63 to -0.39, 50 patients). Besides PEG, lactulose and MOM have been used as second-line drugs, with MOM being very cheap and widely available in some Asian countries such as Thailand[72]. However, the main limitation of MOM is its terrible palatability as opposed to lactulose. The concerning adverse effect of MOM is just only awareness of hypermagnesemia, especially with long-term usage in children with chronic renal disease. Conversely, lactulose can used safely even among preterm infants[73], with the only common adverse effect being abdominal distension. Though PEG is the most effective osmotic laxative for the treatment of function constipation in children, in areas or situations where availability is limited, MOM or lactulose might be used as the standard medication for FC instead of PEG[6,37].

Stimulant laxatives: Stimulant laxatives, such as senna and bisacodyl, increase intestinal motility and interfere with water and electrolyte transport across the epithelial layer. Therefore, stimulant laxatives might result in cramping and abdominal discomfort[1,6,74]. When osmotic laxatives alone are ineffective in treating chronic constipation, stimulant laxatives are often considered. Although stimulant laxatives are thought to be safe and beneficial for treating childhood constipation, limited high-quality RCTs have evaluated their use[66,74,75]. Based on expert opinion, the use of stimulant laxatives may be considered as an additional or second-line treatment[37].

Lubricants: The most popular lubricant laxative is mineral oil, often known as liquid paraffin. Mineral oil works by coating and lubricating stools, lowering fecal water absorption in the colon, and making it easier to pass feces. Given that mineral oil has no chemical activity, severe negative impacts are rarely common. The effectiveness of mineral oil and oral laxatives in treating childhood constipation has been compared in a few low-quality trials. Accordingly, mineral oil promoted significantly greater bowel

movement frequency compared to lactulose[66,76,77], but no significant difference in treatment response was observed when compared to PEG[78]. Moreover, liquid paraffin was found to induce significantly better defecation frequency and FI episodes compared to senna; however, the evidence was of low quality[79]. Given the risk of aspiration and severe lipoid pneumonitis, mineral oil is not recommended for infants under the age of 1 year[80].

Promising pharmacological therapies

Probiotics/prebiotics: Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits to the host. Probiotics have been used in the treatment of FC based on the hypothesis that they alter the intestinal microbiota and colonic pH, thereby improving gastrointestinal motility. Studies reported that constipated children have higher amounts of *Lactobacillus spp.*[81,82] and lower amounts of *Bacteroides*[83] compared to healthy children, implying that gut dysbiosis in the pathogenesis of constipation. So far, however, strong evidence to support the benefits of probiotics in the treatment or prevention of constipation has been limited[84-87]. However, some studies have demonstrated significantly increased stool frequency or softer stools after receiving probiotics. These findings might imply the significant impact of the pathogenesis of stool withholding in constipated children and that probiotics cannot be expected to overcome withholding behaviors. Therefore, future well-designed studies are needed.

5-HT$_4$ receptor agonists: Serotonin controls gut motility, visceral sensitivity, and intestinal secretion through serotonin 5-HT$_4$ receptors, which are primarily expressed by enteric nervous system interneurons[6].

5-HT$_4$ receptor agonists cisapride and tegaserod, which showed similar benefits for treating childhood constipation, were discontinued due to their increased risk for cardiovascular accidents and prolonged QT interval. Prucalopride is a new generation of selective, high-affinity 5-HT$_4$ receptor agonists that stimulate gastrointestinal motility and act primarily on parts of the lower gastrointestinal tract[6,74,88]. Current research on the benefit of prucalopride for constipated children has been contradictory. In an open-label pilot study, prucalopride had favorable effects on stool frequency, stool consistency, and frequency of FI in children with FC[89]. However, a recent multicenter RCT in 213 constipated children found no significant improvement of symptoms compared to placebo[90]. Common side effects include headache, nausea, abdominal pain, and diarrhea.

Chloride channel activators: Lubiprostone is a prostaglandin E$_1$ derivative that activates the chloride channel, thereby stimulating intestinal fluid secretion without increasing serum electrolyte levels[6,74,88]. A study on 127 children (3-17 years old) with constipation showed that lubiprostone was effective and well tolerated, with only minimal side effects like nausea and vomiting[91]. Another study evaluating the safety and tolerability of oral lubiprostone over the course of 24 wk for the treatment of childhood FC in patients aged 6-17 years old showed that lubiprostone was well tolerated and the frequency of treatment-emergent adverse events was similar to that seen in previous clinical trials and adults[92]. However, a double-blind, placebo-controlled, multicenter study on 606 children aged 6-17 years old (202 placebo; 404 Lubiprostone) with FC who satisfied the Rome IV criteria showed no significant difference in the total spontaneous bowel movements response rate between the lubiprostone and placebo groups. Frequently reported side effects include nausea, vomiting, diarrhea, and stomach pain[93].

Linaclotide and plecanatide bind to and act as an agonist of guanylate cyclase-C receptors, causing an increase in the production of chloride and bicarbonate the intestinal lumen. This increase in intestinal fluid causes an acceleration of the gastrointestinal transit while simultaneously decreasing visceral pain by reducing pain sensation[6,74]. Although no pediatric trials have been conducted, studies in adults showed that plecanatide treatment significantly improved constipation. The use of linaclotide in children with FC (0-18 years old) was only reported in a retrospective study, which showed that 45% patients with FC had a positive clinical response and approximately one-third of children experienced negative side effect, such as diarrhea, abdominal pain nausea, and bloating. Eventually, 27% patients stopped using linaclotid due to adverse events[94].

Herbal and traditional medicine: Stool withholding behavior is the main pathogenesis of FC in children. Given that successful toilet training takes time, normally more than 3-6 mo, osmotic laxatives have been the mainstay of treatment during toilet training. However, the majority of guardians and children wanted to withdraw the medication due to their concerns, sometimes with the taste or the amount of osmotic laxatives, which worsened the constipation. In several countries such as Iran, China, Vietnam, and Thailand, herbal medicine is usually integrated into some parts of the treatment as the main or additional therapy. Furthermore, while developing a new drug is a time-consuming and costly process, traditional drugs can be used to treat FC in children instead.

Several herbal and traditional medicines have been used for managing constipation in children, such as glucomannan[95,96], cocoa husk[97], AFPFF (combined acacia fiber, psyllium and fructose)[98], cassia fistula emulsion[99], inulin[100], black stap molasses[101], XiaojiDaozhi Decoction[102], damask roses[103,104], and other herbal medicines[105]. However, only a small number of herbal remedies for

FC have been well supported by RCTs in children (Table 6).

Most studies have reported that herbal and traditional medicine had significant effects on childhood constipation without significant adverse effects. Nonetheless, more high-quality studies are needed. This also suggests the need for studies that identify the active ingredients of herbal and traditional medicine responsible for their beneficial effects against FC in children. With more evidence, herbal or traditional medicine therapies can be integrated into standard treatments for childhood.

Nonpharmacological management

Diet (fiber and water): A low dietary fiber intake has been considered a risk factor for the development of FC[1,6,106]. The recommended adequate dietary fiber intake in children older than 2 years of age is equivalent to age (in years) plus 5-10 g/d[1,107]. However, there is still insufficient evidence from RCTs to support the routine use of fiber supplements to reduce constipation in children[108]. While some studies have shown that constipated children have lower fiber intake compared to healthy controls[96,109], other studies do not support this[95,110]. Regarding to fluid intake, lower water intake has been associated with a higher risk for intestinal constipation[111-113]. Hence, adequate water intake may be beneficial for the prevention of FC. The recommended water intake for children is based on the National Institute for Health and Care Excellence Guideline (Table 7).

Physiotherapy: FC in children is thought to be influenced by dysynergic defecation, which refers to pelvic floor dysfunction[88]. Studies have shown the effectiveness of pelvic muscle exercises as part of a combined treatment intervention[59,88]. A multicenter RCT comparing standard medical care (SMC), which includes education, toilet training, and laxatives, with pelvic physiotherapy (PPT) + SMC in constipated children aged 5-16 years old showed that PPT and PPT + SMC were effective in 63% and 92.3% of the children, respectively. Treatment success (based on the global perceived effect) were achieved in 88.5% and 33.3% of subjects receiving PPT + SMC and SMC, respectively ($P < 0.001$)[114]. A significant study in a primary care environment, however, found no additional benefits of PPT in 134 children aged 4-18[115] and that adding physiotherapy to SMA as a first-line treatment for all children with FC offered no cost benefits compared to SMA alone[116].

Toilet training: Toilet training aims to reduce symptoms, the child's defecation anxiety, and toileting avoidance[1,6]. There are four main methods for toilet training, including the child-oriented toilet training method, Azrin and Foxx method, Dr. Spock's toilet training method, and early elimination toilet training method[117-119]. The most friendly, accepted, and practical methods recommended by the American Academy of Pediatrics and Canadian Pediatric Society is the child-oriented toilet training method, in which the proper age for toilet training is between 18 and 24 mo[120,121]. Parents should be encouraged to be positive and supportive throughout the toilet training. Children should be encouraged to participate in toilet training, which consists of five sequential steps: know, dare, can, will, and do [122]. The child is taught to sit on the toilet for up to 5 min, one to three times a day, following meals to take advantage of the gastrocolic reflex[6]. The position of defecation is necessary to open the anorectal angle (the angle between the longitudinal axis of anal canal and the posterior rectal line, parallel to the longitudinal axis of rectum) and facilitate stool expulsion (Figure 10). To track improvement and compliance, keeping a daily journal of bowel movement, fecal and urine incontinence, and medication is beneficial. Providing stickers or small gifts as positive reinforcement for good behavior might further motivate children[6,122]. Through this process, children gain the ability to perceive their urge to defecate, consequently developing the habit of using the toilet instead of holding it in[59].

Biofeedback: Biofeedback training is a technique for teaching children how to control their perianal muscles for more efficient bowel movements. This technique involves bringing a typically unfamiliar physiological process to the patient's attention and allowing them to measure it[1,6]. In line with this, a recent meta-analysis incorporated three studies contrasting conventional treatment with add-on biofeedback treatment. Accordingly, two studies showed that treatment success rates were higher in the biofeedback group, whereas one study found no difference. In addition, one study found that the addition of biofeedback training at home offered no benefit in terms of defecation frequency compared to biofeedback at the laboratory[106]. Accordingly, biofeedback therapy is not advised for the regular treatment of children with FC based on the most recent research[59,106].

Abdominal massage: The mechanisms by which abdominal massages reduce constipation are most likely a combination of local stimulation and relaxation, as well as stimulation of the parasympathetic nervous system. Direct pressure over the abdominal wall alternately compresses and releases sections of the digestive tract, briefly distorting the lumen size and activating stretch receptors that can reinforce the gastrocolic reflex and trigger intestinal and rectal contraction[106,123,124]. A meta-analysis including a total of 23 RCTs and 2005 children showed that traditional Chinese medicine (TCM) infant massage had a superior effect on infant FC than drug therapy alone. Moreover, a clinical investigation found that children with FC may defecate more frequently and experience less constipation symptoms when receiving TCM infant massage[123]. There is little evidence to support the idea that using Chinese herbs in combination with other therapies might be beneficial[106,123]. Abdominal massage might be a promising additional therapy to manage FC.

Table 6 Characteristics of randomized controlled trials assessing the effects of herbal medications in children with constipation

Ref.	Country	Age (yr)	Study design	No. case (intervention/ control)	Intervention protocol	Probably pharmacological effect of herbal medicine	Duration of treatment/follow-up/end point and outcome measurement	Treatment effect
Esmaeilidooki *et al*[99], 2016	Iran	2-15	Open label, RCT, single center	109 (52/57)	CFE 1 mL/kg per day in three-divided doses (equivalent to 0.1 g of dried pulp of fruits of Cassia fistula). PEG 0.7-0.8g/kg per day	Phenolic antioxidants such as flavonoids, flavan-3-ol derivatives and anthraquinones: Stimulant laxative	Treatment for 4 wk. Primary outcome: Frequencies of defecation, severity of pain, consistency of stool, fecal incontinence and retentive posturing. Secondary: The safety and compliance of therapy	After 4 wk: 86.5% of children in CFE group and 77.1% in PEG group exited from the criteria of FC (RR = 1.121, CI95%: 0.939-1.338). Frequency of defecation that in CFE group (10.96 ± 5.7 stools per week) was significantly more than PEG group (6.9 ± 3.5 stools per week) (P < 0.001). No serious adverse effects in both groups (25% diarrhea and 3.8% abdominal pain)
Cai *et al*[161], 2018	China	1-14	Double-bline RCT, multicenter	480 (120/360)	XEBT: (1) 1-3 years old: 2.5 g, 3 times a day; (2) 4-6 years old: 5 g, 2 times a day; and (3) > 7 years old: 5 g, 3 times a day. Placebo	Seven herbs (Houpo contains magnolol, JueMingZi contain anthraquinones, LuHui contains reactive Aloe-emodin, BaiZhu contains Atractylodes japonica, LaiFuZi, XingRen, ZhiQiao): Promote small bowel peristalsis and work against atropine-induced small intestine suppression in mice	Treatment for 14 daysPrimary outcome: Frequency of SBM for 14 d. Secondary outcomes: Effectual time of defecation, mean symptom scores, disappearance rate of symptoms, recurrence rate and safety outcomes	The mean value of SBM for 14 d were 8.89 and 5.63 in the XEBT and placebo group (P < 0.05). The median effectual time of defecation, main symptom score and disappearance rate of symptoms were significant improved in XEBT group without the significant minor adverse effects between groups
Dehghani *et al* [101], 2019	Iran	4-12	Double-blind RCT, single center	92 (45/47)	BSM (sugarcane extract) 1 mL/kg per day. PEG 1 g/kg per day	The BSM naturally contained polyphenols (960 μg/mL), potassium (12430 μg/mL), iron (10 μg/mL), calcium (3320 μg/mL), zinc (22 μg/mL), sucrose (296000 μg/mL), triterpenoids (11230 μg/mL), phytosterols (7 μg/mL), flavonoids (2 μg/mL and polysaccharides (1250 μg/mL): Polysaccharide act as bulk forming agent, flavonoids/phytosterols and polyphenolic compounds act as natural antioxidants and anti-inflammatory agents	Treatment for 4 wk: Primary outcome: Response rate improvement in frequency of defecations per week, absence of lumpy or hard stools, abdominal pain and retention, soiling and blood-stained stool, sensation of anorectal obstruction/blockage. Secondary outcome: Patients' body weigh was measured in every visit and serological parameters (count blood cells, BUN, creatinine, calcium, phosphorus, sodium and potassium)	Defecation per week was significantly improved in both groups. Symptoms including volitional stool retention, large diameter stool, painful or hard stool and large fecal mass in the rectum decreased significantly two and four weeks after intervention (P < 0.05). No significant difference between the groups. No adverse effects were observed
Qiao *et al*[102], 2021	China	4-14	Double-blind RCT, multicenter study	200 (100/100)	Mixture of 12 herbs[1] (XiaojiDaozhi Decoction) and placebo (5% drug ingredients and 95% dextrin). All received fiber 20 g per day and toilet training)	12.5% *Raphanus sativus L.* (facilitating intestinal motility), 8.33% *Areca catechu L.* (stimulating gastrointestinal cholinergic receptor), 6.67% *Fructus aurantll immaturus* (stimulate gastrointestinal smooth muscle), *Citrus aurantium L.* (stimulate gastrointestinal smooth muscle), 6.67% *Crataegus pinnatifida* (stimulate gastrointestinal smooth muscle), *Magnolia officinalis Rehd* (stimulate gastrointestinal smooth muscle), *Cannabis sativa L.* (moistening the bowel and purging the stools), *Atractylodesmacrocephala Koidz.*	Treatment for 8 wk and follow-up for 12 wk. Primary outcome: Complete SBM (≥ 3 per week) and satisfaction with bowel function. Secondary outcome: Safety and adverse effect (blood measurement of liver and kidney function and lead level)	After 8 wk: 56% of CHM group and 25% of placebo satified with bowel movement (P < 0.05). 40% of CHM group and 19% of placebo had complete spontaneous bowel movement (P < 0.05). No serious adverse effects in both groups

					(stimulate gastrointestinal smooth muscle), *Semen armeniacae amarum* (purgative effect), *Paeonia lactiflora Pall*, *Radix et rhizoma rhei*, (purgative stool)0 and honey (moistening the bowel and purgative effect)		
Tavassoli *et al* [162], 2021	Iran	4-10	Open label RCT, single center	133 (66/67)	Viola flower syrups 5 mL, 3 times a day. PEG 4000 1 g/kg per day	Viola flower contains crude methanolic extract, butanolic and aqueous extracts the stimulate gastrointestinal motility	Treatment 4 wk. Primary outcome: Response off treatment (ROME III criteria). Secondary outcome: Stool consistency, defecation frequency, hard stools, painful defecation, fecal retention, and fecal soiling	Both groups demonstrated significant improvement in stool consistency, number off defecation, hard stool, painful defecation, fecal retention and fecal soiling at the end of the study compared to baseline ($P < 0.001$). No significant difference was observed between the two groups at baseline or at the end of the study ($P > 0.05$)
Nasri *et al* [163], 2021	Iran	2-15	Open-lable RCT	120 (60/60)	LaxaPlus Barij Syrup 1 mL/kg divided into 3 doses. PEG 0.7 g/kg	NA	Treatment and follow-up 8 wk. Primary outcome: Stool consistency, number of defecations, intensity of pain, fecal incontinence. Secondary outcome: Satisfaction rate	After 8 wk follow-up: Bowel movements in the intervention group was significantly higher than in the control group ($P < 0.05$). Pain intensity, and abdominal pain in the group LaxaPlus Barij® decreased significant than control group. No different about satisfaction rate between 2 group
Saneian *et al* [103], 2021	Iran	2-15	Double-blind RCT	60 (30/30)	Goleghand (including honey and Rosa damascene) 0.5 g/kg in three divided dose. PEG: 0.7 g/kg	NA	Treatment and follow-up 8 wk. Primary outcome: The number and consistency of stools per day, painful defecation, abdominal pain, and fecal incontinence. Secondary outcome: Adverse effects and parental satisfaction	After 8 wk: The number of fecal defecations in Goleghand group was higher than PEG ($P < 0.05$). The decrease of defecations after following was more significant in the PEG group than in the Goleghand® group ($P = 0.001$). Parental satisfaction scores did not change in either group ($P > 0.05$)
Imanieh *et al* [104], 2022	Iran	1-18	Double-blind RCT	100 (50/50)	Rosa damascena + brown sugar 1-2 mL/kg (1 mL composed of 0.1 g damask rose and 0.85 g brown sugar). PEG 1-2 mL/kg. All received high fiber diet and hydration	Damask rose: Osmotic laxatives and prokinetic effect. Brown sugar: Osmotic laxatives effect. Possible active ingredients might be phenolic compounds and aqueous fraction (terpenes, glucosides, flavonoids, anthocyanins, kaempferol and quercetin)	Treatment and follow-up 4 wk. Primary outcome: the effective of herbs with PEG. Secondary outcome: Adverse effects	After 4 wk: The cure rate was 100% in the R. damascena group and 91.7% in the control group. Adverse effect of intervention group was the taste which was too sweet

[1]LaxaPlus Barij® Syrup- an herbal medicine that includes *jujube, rose, asparagus, violet flower, borage, quince seeds, and Cordia myxa fruit*. CFE: Cassia fistula emulsion; PEG: Polyethylene glycol; FC: Functional constipation; RR: Risk ratio; RCT: Randomized controlled trial; XEBT: Xiao'er Biantong granules; SBM: Spontaneous bowel movements; BSM: Black strap molasses; BUN: Blood urea nitrogen; CHM: Chinese herbal medicine.

Retrograde enemas: There is insufficient evidence to support the role of retrograde enemas in the maintenance phase of FC in children. Hence, this procedure is reserved for intractable constipation, especially in cases with slow-transit constipation or megarectum. An RCT comparing to the clinical efficacy of supplemental treatment with rectal enemas against conventional treatment alone in 100 children between the ages of 8 and 18 who had symptoms of constipation for at least 2 years revealed that defecation frequency normalized after 1 year of treatment in both groups but was significantly

higher in the intervention group compared to controls at 26 and 52 wk (5.6/wk *vs* 3.9/wk, $P = 0.02$, and 5.3/wk *vs*. 3.9/wk, $P = 0.02$, respectively). Enemas as maintenance therapy for severely constipated children had no substantial side effects compared to oral laxatives alone[125].

Transanal irrigation: Transanal irrigation (Figure 11A) can be an option for children with FC who do not respond to pharmacological treatment[126,127]. It involves inserting a catheter or cone into the rectum to inject water into the colon, cleaning it completely[126]. This treatment has been previously well established for patients with neurogenic bowel disorders and anorectal malformations[127]. Moreover, evidence has suggested its safety and effectiveness, with an average success rate of 78% for both FI and constipation[127,128]. In addition, 86% of the parents were satisfied with the results of transanal irrigation, and 67% reported that they would continue using transanal irrigation for the treatment of their child's symptoms[129]. Therefore, transanal irrigation can be considered an effective therapeutic option for severely constipated children with FI who do not respond to conventional therapy[6,127,128].

Botulinum toxin A injection: Botulinum toxin A (Botox) injections into the anal sphincter can be considered in cases suspected of anorectal dysfunction or functional outlet obstruction[59]. Botox injections, which temporarily reduce anal sphincter muscle contraction, serves as both a diagnostic test, indicating whether the obstructive symptoms are being caused by internal anal sphincter hypertonia, and treatment for intractable constipation. Children who exhibit significant withholding behavior or anal sphincter dysfunction may benefit from Botox injections[6,59]. A RCT and systematic review found that Botox injections were as equally effective as internal sphincter myectomy on short-term follow-up [130,131]. However, a retrospective study on 164 children over 7 years old with intractable constipation showed that Botox injections into the internal anal sphincter of children had an overall response rate of 70%. Moreover, anorectal manometry studies in children with normal and abnormal sphincter dynamics observed similar response rates to this therapy[132].

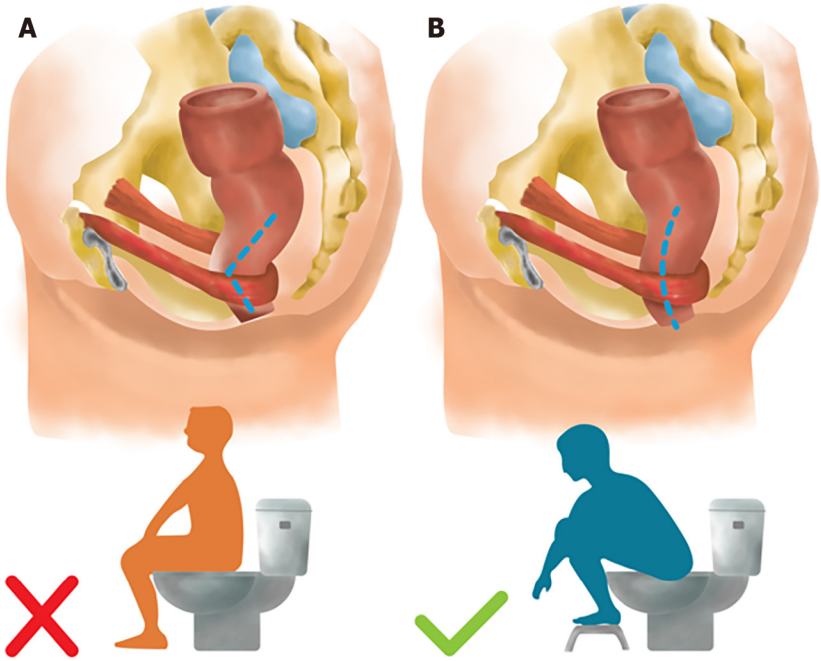
Neuromodulation: Sacral neuromodulation (SNM) is a promising option for the treatment intractable constipation. SNM involves percutaneous placement of an electrode into the third sacral foramen and implantation of a stimulating device under the skin covering the buttocks[6]. The exact working mechanism of SNM remains largely unknown, although evidence has suggested that SNM stimulates anorectal function at a more central level. SNS can affect multiple physiological functions of the pelvis and lower abdomen and supports the propulsive peristalsis of the intestine, which is of special interest in slow-transit constipation[133]. One study in 30 constipated children reported a significant improvement in defecation frequency and abdominal pain after 3 wk of SNM treatment, with the effects being sustained over 22 mo of follow-up in 42% of children. Another study on the treatment of intractable constipation with SNM for over 2 years found that defecation frequency did not change after SNS; however, patients reported that FI decreased from 72% to 20% ($P < 0.01$) and urinary incontinence decreased from 56% to 28% ($P = 0.04$). Minor complications include pain after implantation, displacements of the leads, and infection[134,135]. A recent pilot study that assessed noninvasive SNS in 17 constipated children also found it effective in improving symptoms of constipation[136]. Additionally, abdominal transcutaneous electrical stimulation and posterior tibial nerve stimulation, two skin stimulation techniques, have been used to neuromodulate the bowel to treat constipation with promising outcomes[6].

Surgery: Although almost all patients with FC are successfully treated with conventional therapy, a few continue to have intractable symptoms without any organic problems. In such cases, surgical interventions may be beneficial.

Apart from transanal irrigation, there are also surgical treatment options to achieve stool expulsion, such as antegrade colonic irrigation or antegrade continence enemas (ACEs)[59]. When maximal conventional therapy fails, ACEs have been considered a successful therapeutic option for constipated children[1,6]. ACEs allow fluid to be flushed through the entire colon *via* an external opening into the colonic lumen, which is usually located at the cecum. The most well-established ACE procedures are percutaneous cecostomy and Malone appendicocecostomy (Figures 11B and C). The success rate of ACEs for the management of FC varies from 15% to 100% among studies[130,137,138]. Various enema solutions can be used, including saline and PEG. Complications include skin abrasion, stoma stenosis, granulation tissue, enema fluid leaks, and tube dislodging[137,138]. Regarding other surgical management, there are no clear guidelines on surgical colonic resections and ostomies for children with FC, with such surgical management only reserved for severely constipated children who do not respond to conventional therapy and surgical ACE. These surgical procedures are performed in specialized centers by a multidisciplinary team due to their complexity and potential for problems[6,59].

Table 7 Recommend water intake per day[164]

	Total water intake per day, including water contained in food (mL)
Infants 0-6 mo	700 (water is assumed to be from breast) milk
7-12 mo	800 (milk and complementary foods and beverages)
1-3 yr	1300
4-8 yr	1700
Boys 9-13 yr	2400
Girls 9-13 yr	2100
Boys 14-18 yr	3300
Girls 14-18 yr	2300



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Figure 10 Position during defecation. A: Improper position during defecation; B: Proper position to open the rectoanal angle and facilitate stool expulsion during defecation.

CONCLUSION

FC is the most common FGID in children and it affects the quality of life and psychological health of both the child and the family. Stool withholding behavior is the main etiological agent of FC and successful toilet training is the most effective treatment measure as it also prevents FC recurrence in the long term. Nonetheless, osmotic laxatives and lifestyle modifications, along with adequate fiber and fluid intake, are also crucial as first line therapy during toilet training. Even though extensive history taking and physical examination might enable a diagnosis of FC according to Rome IV criteria, children with intractable constipation may require multiple investigations to confirm the diagnosis or to exclude organic causes. Apart from osmotic laxatives, other promising herbal and alternative therapies have been reported to yield satisfactory outcomes in FC with minimal short-term adverse effects; nevertheless, more evidence is needed before these strategies can be adopted worldwide. Children with intractable constipation typically requires a multidisciplinary team approach and the physician should refer the child to a pediatric specialist for re-evaluation and further management (Figure 12).

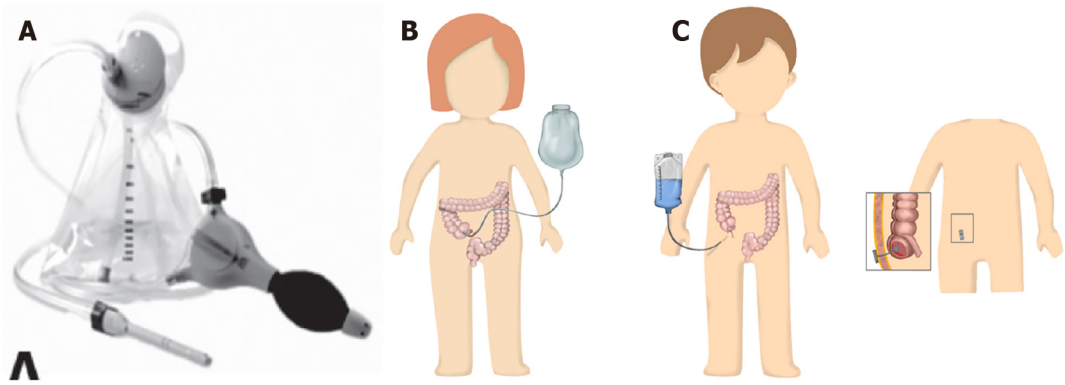
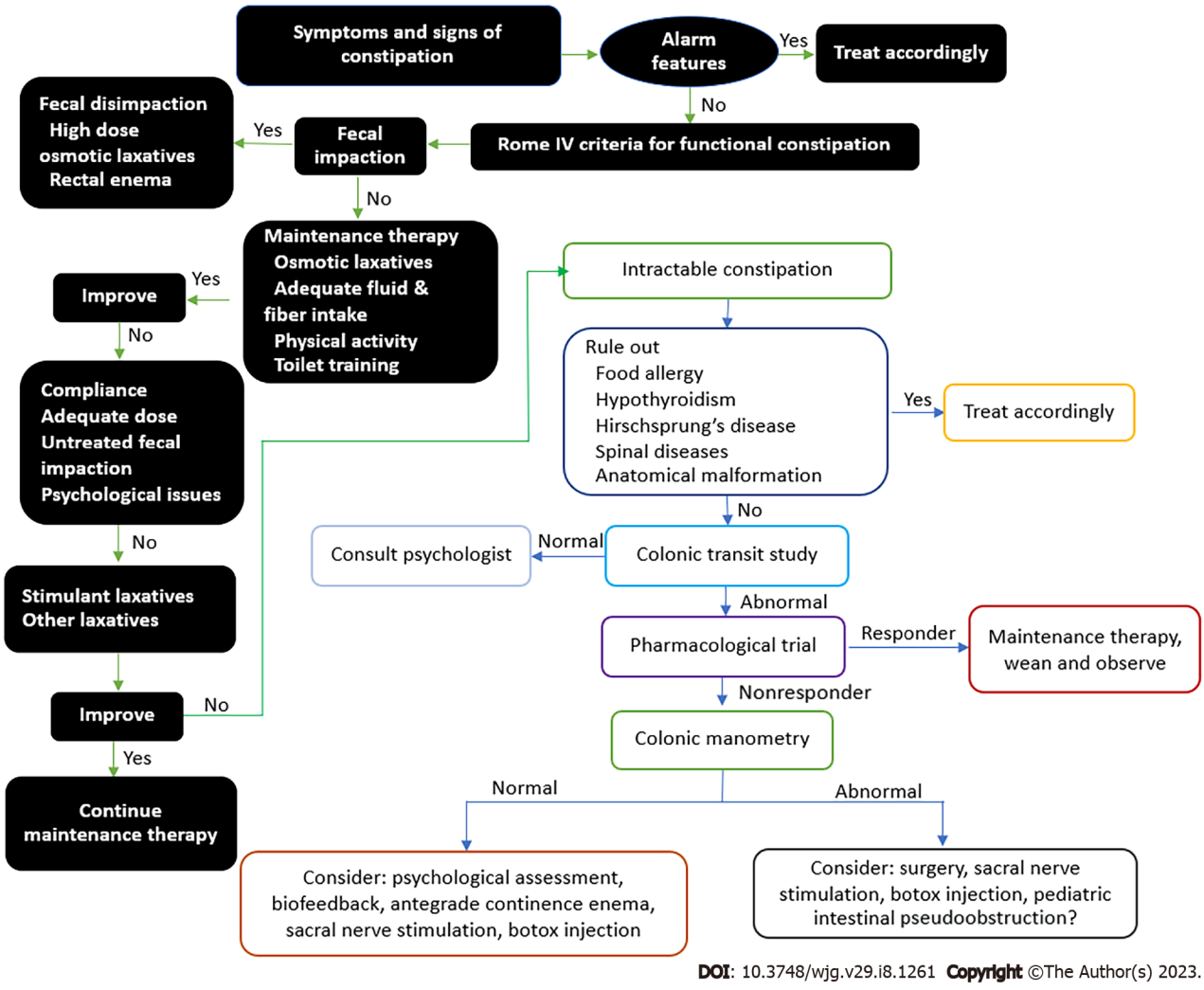


Figure 11 Rectal irrigation method. A: Transanal irrigation; B and C: Antegrade continence enema. A-C: Citation: Palittiya Sintusek. Pediatric lower gastrointestinal motility disorders. In: Werawatganon D, editor. Practical Neurogastroenterology and motility: Basic, testing, and treatment. Bangkok: Printable Inc, 2021: 423-424. Copyright ©The Authors 2021. Published by Thai Neurogastroenterology and Motility Society. The authors have obtained the permission for figure using from Thai Neurogastroenterology and Motility Society (Supplementary material).



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Figure 12 Algorithm for the management of children presenting with signs and symptoms of constipation.

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FOOTNOTES

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Country/Territory of origin: Thailand

ORCID number: Duc Long Tran 0000-0002-2576-9209; Palittiya Sintusek 0000-0003-4441-0151.

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REVIEW

Early-onset colorectal cancer: A review of current knowledge

Margarida R Saraiva, Isadora Rosa, Isabel Claro

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Margarida R Saraiva, Isadora Rosa, Isabel Claro, Department of Gastroenterology, Instituto Português de Oncologia de Lisboa Francisco Gentil, Lisboa 1099-023, Portugal

Corresponding author: Isadora Rosa, PhD, Medical Assistant, Department of Gastroenterology, Instituto Português de Oncologia de Lisboa Francisco Gentil, Rua Prof Lima Bastos, Lisboa 1099-023, Portugal. isarosa@ipolisboa.min-saude.pt

Abstract

Colorectal cancer (CRC) is one of the most prevalent malignancies worldwide. Although most prevalent among older people, its incidence above 50 years old has been decreasing globally in the last decades, probably as a result of better screening. Paradoxically, its incidence in patients below 50 years old [early-onset CRC (EO-CRC)] has been increasing, for reasons not yet fully understood. EO-CRC's increasing incidence is genre independent but shows racial disparities and has been described to occur worldwide. It follows a birth-cohort effect which probably reflects a change in exposure to CRC risk factors. Its incidence is predicted to double until 2030, which makes EO-CRC a serious public health issue. Both modifiable and non-modifiable risk factors have been identified - some are potential targets for preventive measures. EO-CRC is often diagnosed at advanced stages and histological features associated with poor prognosis have been described. EO-CRC presents some distinctive features: Microsatellite instability is common, but another subtype of tumours, both microsatellite and chromosome stable also seems relevant. There are no age-specific treatment protocols and studies on EO-CRC survival rates have shown conflicting data. Due to the higher germline pathological mutations found in EO-CRC patients, an accurate genetic risk evaluation should be performed. In this review, we summarize the current evidence on epidemiological, clinical, histopathological and molecular features of EO-CRC and discuss the contribution of genetics and lifestyle risk factors. We further comment on screening strategies and specific dimensions to consider when dealing with a younger cancer patient.

Key Words: Colorectal cancer; Early-onset; Adenocarcinoma; Hereditary; Birth-cohort effect; Risk factors

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Core Tip: The incidence of early-onset colorectal cancer (EO-CRC) has been surprisingly increasing worldwide and it has become a public health issue. Its clinical, genetic, molecular and histological characteristics suggest that this may be a distinct entity, with a more aggressive behaviour. However, both genetic and environmental risk factors seem to contribute to this observed epidemiological shift in CRC incidence. More evidence is needed in order to clarify EO-CRC aetiology and to develop screening and management strategies.

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INTRODUCTION

Colorectal cancer (CRC), the most common gastrointestinal cancer, is the third most common malignancy and the third leading cause of cancer-related death globally[1]. Once perceived as an elderlies' disease, CRC incidence among the younger population [early-onset CRC (EO-CRC)] has been gaining more relevance. Recent epidemiological data show that, probably due to interventions on risk factors, screening programs implementation and better treatment[2], the incidence and mortality of CRC in patients older than 50 have been decreasing for the past decades[3]. On the contrary, from 1939, when the first case-reports of CRC in the young were published[4], the incidence of EO-CRC has been increasing and is expected to double by 2030[5].

Efforts have been made to characterize EO-CRC, questioning if this is a different entity from CRC in the older. As consensus is lacking, the authors review the existing knowledge, namely epidemiology, risk factors, clinical, molecular and genetic characteristics as well as treatment and overall survival (OS). EO-CRC has become a global health issue and there is a need to raise awareness for it, aiming at lowering the suspicion diagnostic threshold for symptomatic young patients.

EPIDEMIOLOGY

CRC belongs to the top 3 of the most prevalent cancers worldwide, representing, in 2020, 10% of the global cancer incidence and 9.4% of all cancer-related deaths[1,6]. Globally, both CRC incidence and mortality have been decreasing in the last decades[3,5], which is consistently associated to screening programs implementation, thus allowing the detection and removal of adenomatous polyps and consequently interrupting the adenoma-carcinoma sequence[7]. In fact, the inflection time point when CRC incidence began to decrease occurred in the 1990s, coincident with the adoption and diffusion of colonoscopy for screening of average-risk CRC patients in several countries[8].

CRC incidence follows a heterogeneous geographical distribution. The highest incidences have been reported to occur in China, United States of America and Japan. Studies suggest that Human Development Index (HDI) is positively correlated with CRC incidence[9], meaning that countries with high HDI have a CRC incidence about 4-folds higher than low HDI countries. Currently, developed countries show a stabilizing or declining incidence rate whereas developing countries have an increasing incidence rate. The later is probably related to the increase in exposure to CRC risk factors[1], reflecting the adoption of a western lifestyle that accompanies economic transition[10].

Current data implies that CRC is going through an epidemiological change. In fact, based on data from the United States Surveillance, Epidemiology, and End Results program, Galloway *et al*[11] concluded that, although CRC incidence is decreasing by 3.1% per year among people above 50 years old, the incidence of EO-CRC is increasing by 1.4% per year. EO-CRC patients currently account for a percentage that ranges from 0.4% to as high as 35.6% with a median of 7% between the considered reports - such high values should be interpreted carefully as they may reflect institutional biases due to cancer centres that probably receive a high referral of EO-CRC patients[12]. Undoubtedly, CRC has become an increasingly more common diagnosis in younger patients.

One problem potentially leading to controversial research results is the lack of an agreed definition of EO-CRC patients. Some authors use a cut-off of 40 years old[12,13], but most reports in the literature apply a 50 years old limit[10,14,15], this later being consistent with the Amsterdam criteria, that identify patients more likely to have an hereditary predisposition for CRC[16]; 50 years old also coincides with the starting age of most screening programs in the general risk population.

Most studies found no significant difference in gender distribution in CRC[12,17] and the available evidence on EO-CRC prevalence found it to be independent of gender as well[13]. Racial disparities in EO-CRC incidence have however been described, namely with a higher proportion of Black patients,

who also have a worse OS rate[18] - the same occurs for CRC in general in the United States[19]. A recent study, that suggested that the EO-CRC incidence has increased in all racial groups, reported the most elevated rise in Non-Hispanic White People. However, the overall incidence of EO-CRC in the United States remains higher for African Americans[20]. In an Israeli study, racial differences were also found, with the highest EO-CRC incidence in the Arabic population[21].

The reasons for the increased incidence of EO-CRC are not fully understood. Some authors suggest that the common use of colonoscopy is responsible for a proportion of the detected CRCs in young adults. This may have happened as a consequence of the diffusion of colonoscopy as a screening method as well as of an increased endoscopic capacity and efficiency, which may have unintentionally lowered the threshold for performing colonoscopy in younger patients[22].

Another important factor to consider when reflecting about EO-CRC incidence is the globally described existence of a strong birth-cohort effect. This is considered to occur when age-specific incidence rates vary by generation as a result of changes in people's exposure to factors associated to disease risk. This is the opposite concept of period effect, when incidence varies at the same time for all generations[23].

A report based on the incidence of CRC in people from 20 European countries, aged 20 to 49 years old and including data from 1990 to 2016, elucidates on this effect. It showed that the CRC incidence for people aged 30-39 years old started to increase exactly 10 years earlier than in the 40-49 years old group and that, although the CRC incidence also increased for 20-29 years old patients, the study period (> 1980) did not cover the time inflection for that group. This suggests that the turning point possibly occurred before 1990. Additionally, the biggest incidence rise occurred in patients 20-39 years[24], which is consistent with results from an American study[5].

This recent rise in EO-CRC has been observed globally. A report including data from 20 European countries found that EO-CRC incidence has raised significantly in 14 of them, in 5 of them was stable and decreased only in Italy[24]. Another study about EO-CRC incidence among 36 countries from 5 continents reported that EO-CRC incidence was stable in 14 countries, decreased in 3 countries (Austria, Italy and Lithuania) and increased in 19 countries. From these 19 countries, 9 presented an isolated increase in EO-CRC incidence rate - in 5 of them CRC incidence was declining in the older population and in the remaining 4 countries it was stable[17].

Additionally, authors concluded that the most rapid increase in EO-CRC happened in countries where incidence rates were already the highest, such as South Korea[10]. This may be justified by the modernization process that occurred earlier in South Korea in comparison with other Asian countries and the consequently rapid dietary transition that took place following the Korean war[25]. The obesity increase that has been occurring worldwide, both for adults and children, may also play an important role, particularly because Asian countries have been experiencing some of the largest relative increases in body mass indexes[26].

In terms of future predictions, it is expected that the incidence rate for EO-CRC will double by 2030, while in older patients incidence it will probably decline by more than one-third. Meaning that, compared to 2010, when only 4.8% of all colon and 9.5% of all rectal cancers were diagnosed in younger patients, by 2030 they will sum up to 10.9% of all colon and 22.9% of all rectal cancers[5]. These findings explain why EO-CRC is clearly becoming an important public health issue.

RISK FACTORS

CRC is a very heterogeneous disease that results from an interaction between genetic and environmental factors. The majority of CRCs are sporadic (70%), a small proportion of cases are caused by inherited syndromes (5%) and the remaining (25%) have an associated hereditary component, which has not yet been well established and is known as familial CRC[27]. Based on current knowledge, both modifiable and non-modifiable factors may also contribute to EO-CRC.

Modifiable risk factors

There is substantial controversy about results from studies concerning risk factors for CRC, and for EO-CRC in particular, especially concerning preventable risk factors. The majority of data about CRC risk factors is based on older age cohorts' evidence and mainly represents mid- to late-life exposures. But when it comes to EO-CRC, early-life exposures may have a predominant role[28].

One of the most consensual risk factors for CRC is excess body weight. This has been associated with an increased risk for CRC[29] and has become more relevant since obesity prevalence has increased in the last decades[30], paralleling the tendency of EO-CRC[31]. In fact, recent data show that obesity is responsible for a 20% higher risk of EO-CRC[32].

Early-age exposures and weight are also suggested to influence CRC risk. A meta-analysis concluded that higher body fatness at an early age is associated with a higher risk of colon cancer in men and women, the same not applying to rectal cancer[33]. This correlation is probably explained by the effect of proinflammatory cytokines produced by the adipose tissue and by the chronic exposure to hyperinsulinemia and insulin-like growth factor 1 (IGF-I), that may contribute to carcinogenesis[34].

Another consistently associated risk factor for CRC is diabetes mellitus, with an increased relative risk of 30%[35]. In fact, a Swedish study concluded that a diabetic patient reaches the 10-year cumulative risk for CRC that justifies the general screening program 5 years earlier than a non-diabetic patient[36]. Its impact on EO-CRC has already been proved as well[37].

Both alcohol[38] and tobacco[39] are independent risk factors for CRC. A recent study also showed their contribution to EO-CRC, particularly when concomitantly present[40,41]. The protective role of physical activity has been described in different studies that demonstrated an inverse relationship between physical activity and risk of colon cancer. This probably happens due to decreased inflammation, reduced intestinal transit time, decreased IGF-I levels, reduced hyperinsulinemia and modulated immune function, that come with physical activity[42].

Conflicting data exists regarding the role of dyslipidaemia in CRC risk. While some studies suggest a protective one[43], others associate it to a higher risk[44]. The association between diet and CRC has been extensively studied. Some of the proposed elements associated to a higher risk of CRC are processed and red meat[45-47] and sugar sweetened beverages[48] - these are believed to influence gut immune response and inflammation[49]. On the contrary, a diet rich in fiber, dairy, fruits, vegetables [45], fish, beta-carotene, vitamin C, vitamin E, vitamin D and folate has shown a protective effect[41,50].

Aspirin and nonsteroidal anti-inflammatory drugs also exert a dose-dependent protective effect for CRC, through inhibition of the cyclooxygenase pathway[51]. Some of these interactions may account for the older onset/EO-CRC findings[41]. The importance of including fiber in the diet is mainly due to the production of short chain fatty acids during fiber fermentation. These have anti-inflammatory and anti-tumor properties[52] and their levels are inversely related with CRC occurrence[53].

The Western diet, typically rich in red and processed meat and poor in fiber, may be implicated on some of the differences found in incidence among populations[49]. Currently, the increasing incidence of EO-CRC is mostly observed in high-income economies or countries transitioning to a high-income economy and thus adopting Westernized lifestyle habits such as a western diet, weight excess and less activity level - all these may contribute to the increase in EO-CRC[10].

Microbiota has been recently proposed to also play a role in CRC pathogenesis. After comparing the colonic bacterial flora of populations considered to be at high and low risk for CRC[54], differences in microbiota composition were described - this may be a consequence of dietary habits and could harbour a risk for the development of CRC. Besides diet, the use of antibiotics may also affect the microbiota composition and it has been showed that the use of antibiotics, especially when used repeatedly[55] or for long periods during early to middle adulthood[56], is associated with an increased risk of developing CRC. From this point on, further research is needed before a microbiota modifying approach is considered. Current data shows conflicting results on prebiotic and probiotic therapy in CRC patients[57,58]. Based on the already mentioned birth-cohort effect, it seems that modifiable risk factors may play an important role in EO-CRC pathogenesis. The majority of the already known modifiable factors for CRC in general are believed to also apply to EO-CRC[41]. However, conflicting and limited evidence demands more studies.

Non modifiable risk factors

The most consensual risk factor for CRC is a positive family history[17]: First-degree relatives (FDR) have an increased risk of developing CRC, especially when the proband was diagnosed at a younger age. In fact, having a FDR diagnosed under the age of 50 increases the risk of developing CRC by more than three-fold[59]. This risk is also increased for people with more than one affected relative[59], and for family history among distant relatives[60]. Additionally, a history of advanced adenomas or even a family history of any kind of adenomas also increases CRC risk[61,62].

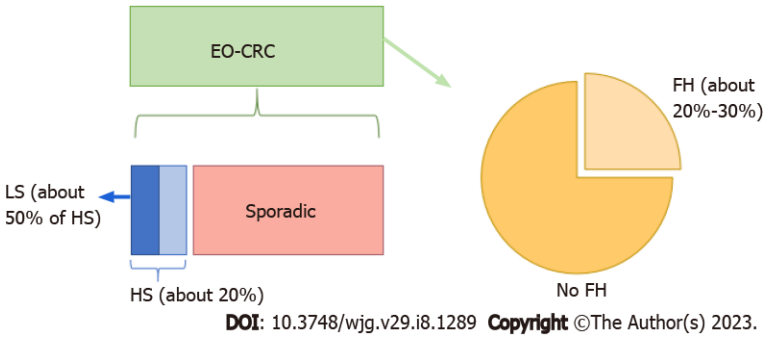
Other known conditions that increase the risk for EO-CRC are Lynch syndrome (LS) and familial adenomatous polyposis, among other hereditary CRC syndromes, with a larger proportion of EO-CRC being hereditary compared with older CRC patients[12,63] (Figure 1). Additional risk of EO-CRC has also been described for patients treated with pelvic radiation at young age[17,64].

Finally, patients with inflammatory bowel disease (IBD) are also known to be at increased risk for CRC[54], due to long-term inflammation. The highest risk is associated with extensive long duration colitis and/or a concomitant primary sclerosing cholangitis diagnosis. The association with EO-CRC, especially for ulcerative colitis, has been clearly demonstrated[15].

Conflicting data has been published about the correlation between breast cancer and CRC. Several studies have suggested an increased risk of CRC for patients with breast cancer at younger ages, and this relationship has also been suggested for EO-CRC[15].

CLINICAL AND HISTOPATHOLOGICAL CHARACTERISTICS

Evidence from different studies found some differences between EO-CRC and older patients CRC, concerning clinical presentation, location and histological characteristics, suggesting it might be a distinct entity. Most studies have agreed on the clinical features of EO-CRC, namely on the most common presentation symptoms, with abdominal pain, rectal bleeding, weight loss, anaemia, decreased



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Figure 1 Genetic risk in early-onset colorectal cancer. HS: Hereditary cancer syndromes; LS: Lynch syndrome; FH: Family history of colorectal cancer; EO-CRC: Early-onset colorectal cancer.

appetite and change in bowel habits[12,52,65], as well as on the more frequent presentation with bowel obstruction[66]. These common clinical symptoms are in accordance with the most common location of EO-CRC in the left colon, more precisely the rectum and the sigmoid colon, as described in the United States[12,23,67-69]. However, global data regarding location are conflicting.

A delay in the diagnosis of EO-CRC has been consistently described, with one series showing an average delay of 4[70] to 6 mo[71] and even with case-reports of a 2 years delay[72]. This represents a 1.4-fold increase in time when compared to older patients[70]. Various reasons can justify this, both doctor and patient related. There is probably both a lower suspicion by the clinicians, hence assuming that the symptomatology is associated to other benign and more common pathology (such as irritable bowel syndrome or haemorrhoids) and consequently not investigating further, and a younger patients' delay in seeking medical attention, out of fear, denial, absence of information about alert symptoms, difficult access to health care or financial reasons[17,72,73].

Several studies consistently demonstrated that younger patients present more often with stage III or IV disease[10,66,74]. In fact, one multicentre retrospective study found that 61.2% of EO-CRC compared to 44.5% of older patients presented metastatic disease at diagnosis[12,13,75]. A plausible explanation for this relates to the absence of screening programs for younger patients but it may also be due to aggressive histopathological characteristics of EO-CRC and to its potential genetic basis, that may predispose to accelerated carcinogenesis in young patients. The diagnostic delay in younger patients may also contribute to more advanced disease at EO-CRC diagnosis[76], although this is not completely explained by the longer time to diagnosis[70].

Furthermore, synchronous or metachronous tumours more frequently arise in EO-CRC[76] but precursor adenomatous lesion are less frequently identified in the EO-CRC groups compared with older ones[13,52] - this also favours the accelerated carcinogenesis hypothesis. Histological predictors of bad prognosis have also been often found in EO-CRC. Young patients' tumours more frequently display adverse histologic features compared with older CRC patients, such as mucinous or signet cell differentiation and poorly differentiated tumours[13,66,68,77]; lymphovascular, venous, and perineural invasion have also been described to be more common[13,66].

MOLECULAR AND GENETIC FEATURES

Since the classical sequence adenoma-carcinoma implicated in the carcinogenesis of CRC has been proposed, our understanding of its biology has evolved and currently CRC and particularly EO-CRC is believed to be an heterogenous disease, including cases with a strong hereditary component as well as sporadic disease[78]. For every CRC patient, a genetic aetiology should be considered, and this is particularly important for EO-CRC patients. In the EO-CRC group, even if we exclude LS (the most common hereditary CRC syndrome) patients, there is still a clear familial component, stronger than for older patients[78].

It is estimated that 20%-30% of CRC are associated with a family history of colorectal polyps or cancer, and that up to 3%-5% of CRC are attributed to an identifiable inherited CRC syndrome, such as LS, familial adenomatous polyposis, MUTYH-associated polyposis, juvenile polyposis syndrome, Peutz-Jeghers syndrome or others[79]. Recent studies concluded that 1 in 5 of EO-CRC cases is attributed to hereditary cancer syndromes[63], and half of those particularly to LS[80] (Figure 1).

Observing mutational profiles of left-sided CRCs, EO-CRC patients were found to have higher rates of mutations in genes related to cancer-predisposing syndromes, such as *MSH2* and *MSH6* (LS), neurofibromatosis type 1, *PTEN* hamartoma tumours syndrome or Cowden's syndrome, tuberous sclerosis complex (*TSC*)*1* and *TSC2*, and *BRCA2* (hereditary breast and ovarian cancer syndrome)[81].

Due to the fact that a larger proportion of EO-CRC seems to be hereditary compared with older patients CRCs, the diagnosis of EO-CRC is currently an indication for referral for genetic evaluation [82]. Additionally, the variability in clinical presentations and potential for phenotypic overlap in CRC justifies the recommendation for genetic testing using a multigene panel for all individuals with an age below 50 years old[83]. This next-generation genetic tests identify pathogenic germline variants in 16%-20% of EO-CRC cases, including genes with moderate to high penetrance in cancer syndromes[63,80].

The diagnosis of a hereditary cancer syndrome frequently influences therapeutical options. In fact, it determines the surgical approach (*e.g.*, subtotal colectomy instead of segmental resection), the chemotherapy regimen choice, colonoscopy surveillance intervals, and management of potential extracolonic cancers[84]. Furthermore, genetic testing is also important to identify relatives that may benefit from anticipation of colonoscopy screening[85].

Although germline genetic alterations can be implicated in 20% of individuals with EO-CRC, hereditary syndromes continue to account for only a minority of cases and the majority of EO-CRC are sporadic, in patients with no family history of CRC[86,87]. CRC represents a heterogenous group of cancers, although its majority can be divided in a few molecular subtypes, that influence treatment options such as chemotherapy regimens and correlate with cancer survival. Interestingly, current evidence suggests a different proportion of molecular tumour subtypes between older and EO-CRCs[68,78,88,89].

CRC usually follows one of the three main molecular subtypes: Chromosomal instability (CIN) or suppressor pathway subtype (80%-85%), CpG island methylator phenotype (CIMP) or serrated pathway (40%) or microsatellite instability (MSI) or mutator pathway subtype (10%-15%). The later is characterized by MSI due to loss of DNA mismatch repair (MMR), occurring both in a sporadic manner or in association with germline alterations in MMR genes (LS), as reviewed in Currais *et al*[84]. These subtypes are not mutually exclusive and can frequently overlap, with a minority of CRCs exhibiting both microsatellite and chromosome stability (MACS)[78,89].

The currently available studies addressing the molecular profile in EO-CRC have reached conflicting results. Some authors present evidence that EO-CRC tumours present more frequently with MSI (MSI-H)[81,90]. MSI tumours in these patients are mostly related to LS[91] and rarely to inactivation of MLH1 (which commonly occurs in older patients) but are more frequently associated with MSH2 inactivation [92]. They are associated with a better prognosis than microsatellite stable (MSS) tumours[93]. Other reports describe that the majority of EO-CRC are rather MSS, lacking DNA repair mechanism abnormalities[94,95]. EO-CRC MSS tumours are typically localized in the left colon, rarely associate with other primary neoplasms and have a strong familial component, features that distinguish them from MSS CRC in older patients[94].

MMR status has potential therapeutical implications. For example, CRCs exhibiting MMR deficiency typically have poor response to fluorouracil-based adjuvant chemotherapy[93]. Interestingly, however, patients with MMR-deficient tumours tend to exhibit better OS, attributed to the more immunogenic nature of these tumours[89]. Additionally, metastatic tumours with MMR deficiency are candidates for immune checkpoint inhibitors[88].

The CpG island methylator phenotype, seems to play a modest role in EO-CRC. EO-CRC are frequently CIMP-low except for EO-CRC LS patients, for whom a higher proportion will be CIMP-high, compared to those who develop LS-related CRC later in life[94]. A more recently described subset of CRC, defined by MACS, because of their diploid DNA content and lack of MMR deficiency, may account for 30% of all sporadic CRCs. These affect younger patients more commonly and have been identified frequently in the left distal colon and rectum. Moreover, this molecular subtype has been associated with poor differentiation, early occurrence of metastasis, disease recurrence and lower survival than patients with MSI or CIN. This can be related to its lack of immune response, opposite to the immunogenic favourable properties of MSI-H CRC[89]. There is still an incomplete understanding of the molecular profile of MACS. Silla *et al*[78] reviewed its main features: MACS tumours are usually CIMP-low, are rarely associated with *BRAF* mutations, lack MLH1 expression, and present a different pattern of hypomethylation than MSI and CIN CRC. Some published studies suggest that MACS may be related to familial CRC syndromes, based on observed increased frequency in young patients[96].

LINE-1 hypomethylation is another feature implied in EO-CRC and is considered to be a surrogate marker for genome-wide hypomethylation. It is associated with increased CIN[97]. Evidence shows that the degree of LINE-1 hypomethylation is an independent factor for increased cancer related mortality and overall mortality in CRC[98]. Antelo *et al*[91] compared this marker in EO-CRC and older CRC and concluded that the first group presented lower levels of LINE-1 methylation, which suggests a distinct molecular subtype of these tumours. Additional studies are needed in order to confirm this association and properly assess the prognostic value of LINE-1 in young-onset CRC.

As for the consensus molecular subtypes classification, EO-CRC tumours are more likely to have subtypes CMS1 or CMS2 tumours compared with CMS3 or CMS4 tumours[68]. From a mutational perspective, despite some conflicting data, the most consensual results show that EO-CRC has lower prevalence of *BRAF* V600, *NRAS*, *KRAS* and *APC* mutations in comparison with late-onset CRC[13,68].

In order to rigorously determine if the described characteristics are sufficient to consider EO-CRC as a distinct molecular profile, more and larger trials are needed. In fact, although lifestyle factors and exposure are believed to contribute to the EO-CRC incidence increase, germline and molecular data also

provide clues to a different CRC pathogenesis.

TREATMENT

EO-CRC does not have specific evidence-based treatment protocols. The subset of EO-CRC patients that present an inherited CRC syndrome, such as LS and polyposis syndromes, should follow their respective guidelines. This is important as the surgical approach is generally more radical and there is a need to manage the risk for other syndrome related malignancies by appropriate screening[3,99].

For the majority of the patients, although there are no age specific recommendations, current studies show that different therapeutic strategies are being implemented according to the patient's age. Surgery is the main curative treatment for CRC, but for high-risk stage II and stage III there is a role for adjuvant chemotherapy[100]. When comparing EO-CRC with an older cohort (65-75 years old), various studies have found that more patients with early-stage EO-CRC are treated with adjuvant therapy for stage II and III disease. In fact, stage II low-risk EO-CRC patients receive adjuvant therapy 50% of the time, compared with 19.1% in the older cohort[101,102]. Curiously, these regimens were not associated with increased survival in stage I or stage II cancers and offered only marginal benefit in stage III and stage IV diseases[102].

Additionally, EO-CRC patients have better chances of receiving surgical treatment either for early-stage or for metastatic disease[66,103], of receiving radiotherapy at all disease stages and also of being offered more aggressive adjuvant treatment, including multi-agent chemotherapy[102]. Another study found that EO-CRC patients with stage I or II were more likely to receive adjuvant or neoadjuvant therapy, compared to older patients[66].

These studies may reflect the fewer comorbidities of EO-CRC patients, a better performance status and fewer adverse reactions to systemic treatment leading to a better tolerance to multiagent regimens than the older patients[66]. However, they may also show a bias towards offering more treatment to younger patients and there is a question of whether they are being victims of overtreatment with no proven benefit. According to current knowledge, equal treatment should be offered to EO-CRC (without a hereditary syndrome). More studies on the putative molecular differences between EO-CRC and older age CRC may in the future bring new molecular treatment targets, allowing specific treatment[11].

SURVIVAL

Some authors, supported by population-based studies, suggest that EO-CRC presents a better survival at every stage[77,103] and even that 5 years after curative treatment, EO-CRC survivors present the same main causes of death than the general population[104]. Other studies demonstrated that, after adjusting for stage, the prognosis is not influenced by CRC age[13,66], with no difference in stage-specific 5-year disease free survival and OS rates[66].

On the contrary, there also reports from single institution studies that showed poor clinical outcomes for EO-CRC and some authors suggest that the worse survival described for EO-CRC is caused by adverse histopathological features[105,106]. O'Connell *et al*[12]'s review found the average overall 5-year survival for young patients to be 33.4%, consistent with other reports[107,108]. When comparing stage-for-stage survival for younger *vs* older patients with CRC, it seems that young patients with earlier stages have better survival than older patients with the same stage disease. On the contrary, EO-CRC patients with advanced stages do the same or worse than older patients with the same advanced stage. This may justify the described overall lower 5-year survival for EO-CRC because these patients frequently present advanced disease, possibly as a consequence of the delayed diagnosis and/or the more aggressive underlying nature of the disease[12].

Regarding advanced and metastatic disease, studies found that EO-CRC patients have a lower progression-free survival although this does not impact the relative risk of death or OS compared with older CRC patients[109]. As for the mortality rate, although CRC mortality is declining overall and, from 2000 to 2014, CRC death rates decreased by 34% in individuals older than 50 years old, it increased by 13% in EO-CRC patients[110]. One limitation of the studies is that survival outcomes for EO-CRC are often confounded by the inclusion of hereditary colorectal carcinomas, such as LS related CRCs, that are believed to present a better survival compared with non-syndromic colorectal carcinomas[111,112].

SCREENING

CRC incidence and mortality for people older than 50 years old have been declining and the main proposed reason for that is the successful implementation of CRC screening, with a possible contribution of a change in lifestyle. Screening programmes have mostly emerged over the past few decades and generically apply to adults starting from 50 to 60 years old[113]. A recent study shows that from 36

countries from 5 continents, only 4 (Costa Rica, Cyprus, India and Philippines) lack any form of screening programmes[10]. Additionally, only 4 from the 36 mentioned countries have an earlier age cut-off for screening: Italy, beginning at 44 years; China, Japan and Austria, where it begins at 40 years old[113].

On the contrary, prevalence of EO-CRC is increasing. Although one reason for this increase could be over detection through screening, that seems unlikely since screening before age 50 is rare in the general population in most countries[113]. This was the reality until the most recent publication of the American Cancer Society (ACS) guidelines that, for the first time, recommend starting CRC screening at 45 years old for the standard-risk population. The previous CRC screening recommendations have been based on randomized controlled trials and prospective cohort studies. Since there is a lack of data on screening below 50 years old, this recommendation was a result of simulation modelling analyses of screening outcomes, that showed a favourable balance between benefits of screening from 45 years old with its potential life-years gained and the related burdens[114].

Although the absolute risk for EO-CRC is still low, it is expected to increase and the disease burden for younger patients is already substantial and potentially long-term. Additionally, current data show that 40-45 years old adults have the same prevalence of adenomas - CRC precursor lesions[115] - than adults 50-54.5 years old and people aged 40-49 years old account for 75% of the EO-CRC patients[116]. This questions the current age threshold and supports the ACS recommendation.

The debate about lowering the screening age is ongoing, as reviewed in Anderson and Samadder [117]. Those who are favourable to this approach prioritize the need to control the increase in EO-CRC. A study on screening worldwide, showed that two (Austria and Italy) of the only three countries where EO-CRC has declined have screening programs that begin below 50 years old (Italy at 44 and Austria at 40)[113]. Curiously, in Austria, the decreasing incidence of EO-CRC has only occurred in the 40 to 49 years age group[10]. Although another study showed that the same happened in Italy, other described an incidence decrease among the 20-39 years old patients[24].

Reasons for opposing the new 45 years old age threshold are diverse. One argument is that studies have shown that the biggest increase rate in EO-CRC was observed for people aged 20 to 39 years old, an age sector still not included in the suggested screening program. Another argument is that, although increasing, the absolute numbers of EO-CRC are still low, not relevant enough for a screening strategy change. Other concerns are related to the risk of augmenting health inequities related to access to health care services as well as for excessive financial health care costs, especially considering most European countries are still struggling to finance the current screening programs or are still in the process of implementing them[24].

Other authors suggest waiting for adequate randomized screening studies to test the ACS recent recommendation before adopting it and even question if the screening strategy should be applied to a malignancy whose biological and molecular substrate is not fully understood[95], which could mean that standard screening methods and prevention may not even be appropriate for it.

Another small study, based on the evidence that EO-CRC affects predominantly the left side of the colon, suggests a screening strategy with flexible sigmoidoscopy to be applied to people from 40 to 49 years old, as their study group observed that 80% to 83% of those tumours were theoretically within the reach of a flexible sigmoidoscopy[67]. From a different angle, another important factor when reflecting on EO-CRC diagnosis is that only half of the EO-CRC patients with germline pathogenic mutations reported a CRC diagnosis in a FDR and, consequently, were not eligible for high-risk CRC screening [63]. This contributes to the recommendation of performing genetic testing in all EO-CRC. Moreover, tumour testing for MSI or immunohistochemistry for MLH1, MSH2, MSH6, and PMS2 is recommended for all EO-CRC patients, even in countries where universal CRC MSI screening is still not implemented [3].

Another important aspect is the need to alert the medical community for the increasing incidence of EO-CRC and the consequent need for a rigorous assessment of CRC family risk trough detailed and complete family history in medical records, namely reviewing the familiar history of cancer including first- and second-degree relatives as well as personal and familiar history of advanced polyps[82]. Such systematic evaluation may be difficult to reliably execute in real world clinical practice, but it allows an appropriate determination of the patient risk for CRC and the identification of high-risk patients who should be offered earlier screening. Additionally, existing genetic risk models can also be employed and help identifying patients that benefit from genetic testing[118].

Another debatable option is to offer EO-CRC patients next-generation sequencing technology for genetic germline testing. Multigene panel testing has increased detection of germline mutations[79] in a feasible and more cost-effective way than single gene testing[82]. Recently, the National Comprehensive Cancer Network guidelines proposed offering multigene panel testing to all EO-CRC, aiming to increase opportunities for primary and secondary cancer prevention[83].

The disadvantage of this approach is that by testing more genes there is a higher risk of finding genetic variants of unknown significance or without a clear management evidence-based guideline[3]. As so, a multidisciplinary discussion with the active participation of a genetics specialist is recommended to appropriately offer and interpret genetic results, hence providing an appropriate counselling to EO-CRC patients and their families[3].

SPECIFIC CONSIDERATIONS IN THE YOUNG

When dealing with younger patients with a malignancy, there are some extra needs that should be remembered and appropriately managed. EO-CRC patients, as older ones, may need to deal with adverse effects of the different treatment modalities. They face a higher rate of sexual dysfunction than the general population and report to be less sexually active after surgery[119]. Although surgery for CRC does not generally affect fertility[3], chemotherapy, particularly fluorouracil, may reduce sperm count or cause amenorrhea and radiotherapy may also reduce fertility in both men and women[120].

Fertility may play a central concern in the management of EO-CRC patients since they may still be in the reproductive age and have incomplete reproductive plans. As so, patients should be referred to reproductive specialists because options for fertility preservation such as banking of cryopreservation of sperm/oocytes/embryos is recommended prior to gonadotoxic chemotherapy and should be discussed, ahead of starting treatment[121].

Other aspect to consider in EO-CRC patients is the possibility of the diagnosis and/or treatment to be coincidental with a pregnancy, especially with the common delay in childbearing age in the developed world. In that scenario, a multidisciplinary approach, weighting the risks and benefits for both the mother and the foetus should be employed. Although studies in pregnant women are missing, it is consensually agreed that the first trimester is the most vital period to avoid, if possible, systemic agents [122]. Besides fertility, another concern for EO-CRC is to cope with the eventual need for transient or permanent ostomy. The present or past history of an ostomy can contribute to worse sexual function and body image[123].

Additionally, EO-CRC patients, especially the ones submitted to multimodality therapy, will live the rest of their lives under the threat or burden of long-term sequelae. Long-term survivors have reported sustained functional deficits and symptoms, such as sexual disfunction, anxiety, worse body image, embarrassment with bowel movements and faecal incontinence[124], with an impact on their quality of life. Concerning the psychosocial dimension, higher depression and anxiety diagnosis have been described for CRC survivors[125]. Concordantly, the American Society of Clinical Oncology recommends that all cancer survivors should be periodically evaluated for symptoms of depression and anxiety using validated measures and adequately managed, if necessary with the referral for psychological or psychiatric consultation[126]. All of these afore-mentioned factors contribute to the EO-CRC burden of disease, diminish patients' quality of life and should be appropriately addressed throughout patients' life.

CONCLUSION

By 2030, previsions are that more than 1 in 10 colon cancers and 1 in 4 rectal cancers will be diagnosed in patients younger than 50 years old[5]. These numbers are alarming and, consequently, as EO-CRC incidence increases, many studies have tried to shed light on this entity, especially comparing it to the traditional older patient CRC. Evidence suggests that EO-CRC differs from older-onset CRC in its clinical characteristics, pathological features, aggressive behaviour, and molecular profiles. These may be responsible for different responses to treatment and survival, although further studies are needed.

Although a higher proportion of cases, comparing to older patients, is genetic or familial, the majority of EO-CRC cases is still probably related to somatic genetic mutations or epigenetic alterations induced by modifiable risk factors. This is consistent with the birth-cohort effect observed. The contribution of lifestyle risk factors stands as an opportunity for implementing primary prevention measures for CRC, such as the practice of physical activity, having a healthy diet, quitting smoking and limiting alcohol consumption, also preventing obesity and diabetes. A study showed that risk factor modification could diminish CRC-related mortality in about 12% over 20-years[2].

This paper also aims at raising awareness of all the clinicians for the need to lower the suspicion threshold for young patients presenting with alarming gastrointestinal symptoms. We also stress the need to obtain complete clinical information, such as history of polyps, familial history (first- and second-degree relatives) and personal diseases history, namely previous radiation therapy or IBD. Only with this information can the genetic risk of EO-CRC be properly evaluated and subsequent genetic consultation be asked for, when indicated.

EO-CRC patients have some peculiarities and clinicians should be conscious of them. Financial burden and treatment sequelae such as sexual dysfunction, incontinence, anxiety and depression must be considered and managed. Patient's reproductive plans should also be discussed. To conclude, although currently we already have some important data about EO-CRC, larger studies with longer follow-up of these patients are needed in order to reach consensus about the nature and pathogenesis of this disease.

Namely, ongoing studies and clinical trials, by our group and several others across the world, on the impact on survival of increasing clinician awareness on EO-CRC, on the definition of potentially specific phenotypes of EO-CRC, or on their specific responses to radio-, chemo- or immunotherapy will shed light on the subject. Hopefully, by improving young patients' diagnosis, diminishing its observed delay,

tailoring its treatment and follow-up and implementing modifications in lifestyle risk factors, in the future EO-CRC incidence will follow CRC incidence pattern and both entities' burden will decrease.

FOOTNOTES

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Country/Territory of origin: Portugal

ORCID number: Margarida R Saraiva 0000-0002-6750-1112; Isadora Rosa 0000-0002-2953-5987; Isabel Claro 0000-0002-1860-6161.

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MINIREVIEWS

Hamartomatous polyps: Diagnosis, surveillance, and management

Leva Gorji, Peter Albrecht

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Leva Gorji, Peter Albrecht, Department of Surgery, Kettering Health Dayton, Dayton, OH 45405, United States

Corresponding author: Leva Gorji, DO, Doctor, Department of Surgery, Kettering Health Dayton, 405 West Grand Avenue Dayton, Dayton, OH 45405, United States.
leva.gorji@ketteringhealth.org

Abstract

Hereditary polyposis syndrome can be divided into three categories: Adenomatous, serrated, and hamartomatous polyps. Hamartomatous polyps, malformations of normal tissue presenting in a disorganized manner, are characterized by an autosomal dominant inheritance pattern. These syndromes exhibit hamartomatous gastrointestinal polyps in conjunction to extra-intestinal manifestations, which require conscientious and diligent monitoring. Peutz-Jeghers syndrome, Cowden syndrome, and juvenile polyposis syndrome are the most common displays of hamartomatous polyposis syndrome (HPS). Diagnosis can be pursued with molecular testing and endoscopic sampling. Early identification of these autosomal dominant pathologies allows to optimize malignancy surveillance, which helps reduce morbidity and mortality in both the affected patient population as well as at-risk family members. Endoscopic surveillance is an important pillar of prognosis and monitoring, with many patients eventually requiring surgical intervention. In this review, we discuss the diagnosis, surveillance, and management of HPS.

Key Words: Hamartomatous polyps; Peutz-Jegher syndrome; Cowden syndrome; Juvenile polyposis syndrome

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Core Tip: We hope that our review article, "Hamartomatous polyps: Diagnosis, surveillance, and management", will function as a concise review of the literature as pertaining to the diagnosis, surveillance, and treatment of the most common hamartomatous polyp syndromes.

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INTRODUCTION

The national cancer institute defines a harmartoma as, "a benign growth made up of an abnormal mixture of cells and tissues normally found in the area of the body where the growth occurs"[1,2]. Diagnosis may be pursued based on personal or family history, endoscopic evaluation, and histopathology identified on endoscopic tissue sampling. The most common disease processes associated with hamartomatous polyps include Peutz–Jeghers syndromes (PJS), Cowden syndrome (CS), and juvenile polyposis syndrome (JPS). While hamartomatous polyps themselves are benign, diligent surveillance is imperative as a significant risk of malignant transformation exists. Additionally, the disease process is often associated with an increased risk of other additional malignancies. As a result, management often requires multidisciplinary evaluation[1,3]. In this review, we discuss the diagnostic considerations, clinical manifestations, gastrointestinal (GI) and extra-intestinal surveillance recommendations, and management options of the most common syndromes associated with hamartomatous polyps.

PJS

Diagnosis

PJS is an autosomal dominant mutation of the mammalian target of rapamycin (mTOR) pathway as a result of a germline mutation of the serine-threonine kinase (*STK11/LKB1*) tumor suppressor gene[4]. The *STK11/LKB1* gene is located on chromosome 19p13.3 and plays an important role in the secondary messenger pathway, which modulates cellular proliferation, responds to cellular energy deficits, and controls cellular polarity[5-7]. The incidence of PJS is estimated to range between approximately 1:8300 and 1:200000 with nearly 70% of cases demonstrating familial inherence and 30% of cases arising from a sporadic mutation[3,8,9]. Although there is often variable penetrance and clinical heterogeneity, diagnostic clinical criteria include: Any number of polyps with a family history of PJS, two or more Peutz-Jeghers polyps (PJPs), mucocutaneous pigmentations with family history of PJS, or any number of PJPs with mucocutaneous pigmentation[4,8,10]. While endoscopically similar to other polyps, PJPs have a characteristic phyllodes appearing epithelial component and a cystic glandular component which extends into the submucosa and muscular propria on hematoxylin and eosin staining[7,11-13]. Notably, PJPs are exclusively located within the small bowel. Gastric polyps associated with PJS have similar characteristics to hyperplastic gastric polyps and are not considered PJPs[7,12]. The median age of initial polyp development is 12 years-old, with nearly half of patients experiencing symptoms by the age of 20 [7,14]. The primary clinical manifestation of PJS is chronic bleeding from GI polyps leading to anemia. Small bowel obstruction secondary to an intussusception from a hamartomatous polyp of the GI tract occurs in up to 70% of patients, with the average age of diagnosis being 23 years-old[7]. Patients also characteristically present with mucocutaneous hyperpigmentation surrounding the oral cavity, eyes, nostrils, or surrounding the anus[15].

Surveillance

Due the increased risk of GI and extra-intestinal malignancies, a multidisciplinary approach to screening is encouraged. While the mean age of index cancer diagnosis is 41 years old, PJS is associated with an overall cumulated malignancy rate of 90% by the age of 70[4,8]. GI malignancies commonly associated with PJS include gastric, intestinal, and the pancreatic. Additional extra-intestinal malignancy manifestations include breast, endometrial, ovarian, and lung[11]. Multiple studies have been pursued in order to further characterize the malignancy risk that is associated with PJS (Table 1). The National Comprehensive Cancer Network, European Hereditary Tumour Group, and the American College of Gastroenterology (ACG) provide surveillance recommendations for patients[16-19]. A meticulous regimen of lifelong malignancy surveillance is imperative (Table 2).

Management

Endoscopy is an essential modality of surveillance and management of polyps associated with PJS. Polypectomy during esophagogastroduodenoscopy (EGD) and colonoscopy is particularly appropriate for patients with a limited number of polyps ranging from 0.5-1 cm in size. If the polyposis that is encountered is too large or numerous to be managed endoscopically, surgical resection of the diseased segment may be pursued[18,20].

Table 1 Studies investigating the malignancy risk associated with Peutz-Jeghers syndrome

Ref.	*N*	Location	Outcome
Chen *et al*[80]	336	China	Patients with PJS possess a 50% cumulative malignancy risk by the age of 60, with the most common malignancy being colorectal cancer at a median age of 41
Hearle *et al* [81]	419	United Kingdom	The risk of developing a malignancy is 85% at the age of 70 in patients with PJS, most common being gastrointestinal in origin
Ishida *et al* [82]	583	Japan	Patients with PJS possess a cumulative malignancy risk of 83% by the age of 70, with an increased rate of gynecologic malignancy in comparison to previously reported data
Korsse *et al* [83]	144	Netherlands	The cumulative risk of pancreatico-biliary malignancy is 32% by the age of 70 in patients with PJS
Mehenni *et al* [84]	149	Switzerland	Patients with PJS have a cumulate malignancy risk of 67% at age 70, particularly with *STK11/LKB1* mutations in exon 6. Malignancies most commonly occur in the GI tract
Resta *et al*[11]	119	Italy	The *STK11/LKB1* mutation is associated with a relative overall cancer risk of 15.1, with pancreatic and cervical malignancies being the most common; median age of diagnosis noted to be 41 yr
Van Lier *et al* [8]	133	Netherlands	Patients with PJS possess a cumulative malignancy rate of 76% by the age of 70; malignancies most commonly occur in the GI tract and in women

GI: Gastrointestinal; PJS: Peutz-Jeghers syndrome.

Table 2 Malignancy screening for Peutz-Jeghers syndrome[4,8,10,16-18,85,86]

Malignancy	Screening
Breast	Annual self-breast exam beginning at the age of 18. Annual breast exam with MRI or mammography beginning at the age of 25, and as needed
Cervical	Cervical smear annual beginning at the age of 20, and as needed
Colon	Colonoscopy every 1-3 yr beginning at the age of 8, and as needed. Routine surveillance at 18 if baseline endoscopy at age 8 is negative
Pancreas	Annual endoscopic ultrasound or MRCP/ERCP beginning at the age of 30, and as needed
Stomach	EGD every 1-3 yr beginning at the age of 8, and as needed. Routine surveillance at 18 if baseline endoscopy at age 8 is negative
Small bowel	Capsule endoscopy or CT/MRI enterography every 2-3 yr, beginning at the age of 8, and as needed
Testicular	Annual testicular exam and ultrasound beginning at birth
Uterus	Annual pelvic ultrasound and exam beginning at the age of 25, and as needed; consider total hysterectomy once complete with child-bearing

MRI: Magnetic resonance imaging; CT: Computed tomography; MRCP: Magnetic resonance pancreatography; ERCP: Endoscopic retrograde cholangiopancreatography; EGD: Esophagogastroduodenoscopy.

Management of small bowel polyps includes double balloon enteroscopy (DBE) or surgery. DBE allows for diagnosis and treatment of polyps located in the jejunum and the ileum. The device possesses a 200 cm long enteroscope with a 145 cm long overtube. There are two latex balloons attached to the tip of the endoscope and overtube, which are managed with a pressure-control pump system serving to prevent redundancy of the small bowel[21-23]. With this approach, manipulation and advancement of the scope may be occasionally impaired in patients who have previously undergone abdominal surgery secondary to intraabdominal adhesions, warranting intraoperative enteroscopy[24,25]. The European Society of Gastrointestinal Endoscopy (ESGE) recommends elective polypectomy for small bowel polyps that are > 15-20 mm in size to reduce the risk of intussusception. In symptomatic patients, polyps that are < 15 mm should be removed, as well[19]. Complications from DBE occur in less than < 1% of cases, but include perforation, post-procedure hemorrhage, pancreatitis and aspiration[23,26]. DBE is an effective method for early detection and non-operative removal of polyps located in the small bowel[18,27]. Similar to polyposis of the colon, surgical resection may be required in the setting of numerous or large polyposis of the small bowel[19].

Surgical intervention is required for patients with PJS who present with intussusception, a phenomenon where the proximal segment of bowel telescopes into a distal segment of bowel with the polyp acting as a lead point. If no intervention is pursued, bowel ischemia and perforation may occur [4]. Patients with PJS possess a cumulative risk of intussusception reaching nearly 44% by the age of 10,

which increases to nearly 50% by the age of 20[20]. The risk of intussusception is greatest with polyps that are 15 mm or larger[28]. Intraoperatively, frankly ischemic bowel evidently must be removed; however, polypectomy without bowel resection is recommended in cases of reversible ischemia. As the risk of recurrent intussusception is present especially in younger patients, some authors have recommended further evaluate the bowel through manual palpation or enteroscopy *via* an intraoperative enterotomy in order to remove any additional polyps greater than 15 mm[20,29].

According to the ESGE, pancreatic lesions should be addressed with partial pancreatectomies in the case of: (1) Solid lesions ³10 mm unless known to be benign; or (2) intraductal papillary mucinous neoplasms with high-risk features including mural nodules, enhanced solid component, obstructive symptoms, enhanced cyst wall, abrupt changes to duct size, and main pancreatic duct ³10 mm[19,30].

Breast lesions should be managed based on current breast cancer guidelines, as no therapeutic strategy specific to PJS is presently available. A mastectomy for the purpose of risk reduction should be discussed in a multi-disciplinary setting accounting for additional patient risk factors and family history [19]. Similarly, while an established malignancy risk exists between PJS and cervical, uterine, and testicular cancer, no formal intervention guidelines specific to the correlation exists.

Studies have also demonstrated that the immunosuppressant agent Sirolimus (Rapamycin) has led to reduction in the number and size of polyps in a mouse model, which is largely attribute to the anti-angiogenic properties of the drug[31-33]. Currently, phase 4 prospective study for characterization of the drug in patients with PJS is being undertaken.

CS

Diagnosis

CS is an autosomal dominant mutation in the *phosphatase and tensin homolog* (*PTEN*) gene impacting the downregulation of the mTOR pathway. Consequently, the *PTEN* regulation of cellular proliferation and survival is impaired[34,35]. The incidence of CS is estimated to be roughly 1:200000[36]. CS characteristically presents with hamartomatous polyps which may appear in any organ. Nearly all patients exhibit some type of skin lesion including trichilemmomas, acral keratoses, and papillomatous papules. Additionally, nearly 80% of patients demonstrate GI hamartomatous polyps and 80% of patients exhibit macrocephaly. Diagnosis is based on the guidelines established by the International CS Consortium: Patients must possess two major criteria one of which must be either macrocephaly or Lhermitte-Duclos disease (LDD), one major criteria with three minor criteria, or four minor criteria (Table 3)[37]. 40% of CS is associated with LDD-a rare, slow growing dysplastic gangliocytoma of the cerebellum manifesting with a broad range of clinical findings. The presentation of LDD may range from being asymptomatic to exhibiting signs of ataxia, cranial nerve palsy, vertigo, mental impairment and deterioration, intracranial hypertension, and hydrocephalus. The wide spectrum of clinical manifestations is attributable to the slow growing nature of the neoplasm[38,39].

Surveillance

The majority of patients with CS are at risk for developing breast, thyroid, renal, endometrial, colorectal, and skin malignancies (Table 4). Given this established predisposition, attentive multidisciplinary surveillance and screening is an essential component of disease management (Table 5). The purpose of these surveillance recommendations is early detection in order to allow curative oncologic treatment.

Management

There is no available curative treatment for CS. Rather, management is geared towards early detection of malignancy and timely oncologic treatment. Genetic counseling is also prudent in order to promote early detection and surveillance for kindred. Options for treatment of cutaneous lesions include, surgical excision, 5-Flurouracil, carbon dioxide laser treatments, isotretinoin[40,41]. Polyps found on endoscopy may be removed endoscopically, if amenable. Otherwise, identified malignancies should be addressed utilizing the most up-to-date therapeutic regimens.

Associated syndromes

PTEN hamartoma tumor syndrome (PHTS) is a broad category of hamartomatous pathologies arising as pathogenic *PTEN* mutation variants identified by gene-targeted testing and comprehensive genomic testing. PHTS classically includes CS, Bannayan-Riley-Ruvalcaba Syndromes (BRRS), PTEN-related Proteus Syndromes (PS), and *PTEN*-related Proteus-like syndromes (PLS). Notably, however, segmental overgrowth lipomatosis arteriovenous malformation epidermal nevus (SOLAMEN) syndrome also shares the same mutation[42,43]. It has been proposed that CS and BRRS may simply be varying spectrums of the same syndrome[44]. While formal diagnosis criteria are not present for BRRS, patients often present with macrocephaly, penile mucocutaneous lesions, delayed psychomotor development, and visceral hamartomas[44,45]. Approximately half of patients with BRRS have GI polyposis, with an analogous histopathologic presentation[44]. Nearly 60% of patients with BRRS possess a germline *PTEN*

Table 3 Major and minor criteria for the diagnosis of Cowden syndrome[37]

Major criteria	Minor criteria
Breast malignancy	Fibrocystic changes of the breast
Lhermitte-Duclos disease	Fibromas
Macrocephaly	Gastrointestinal hamartomas
Thyroid malignancy	Genitourinary tumors or malformations
	Lipomas
	Mental retardation
	Thyroid lesions, *i.e.* goiter

Table 4 Studies investigating the malignancy risk associated with *phosphatase and tensin homolog* mutation

Ref.	*N*	Location	Outcome
Tan *et al*[87]	3399	North America, Europe, and Asia	Patient with *PTEN* mutations possess a lifetime breast cancer risk of 85%, thyroid cancer risk of 35%, renal cell carcinoma of 35%, endometrial cancer risk of 28%, colorectal cancer risk of 9%, and melanoma risk of 6%
Fackenthal *et al*[88]	2	United States	There is an increased risk of male breast cancer in patient with *PTEN* mutations and CS
Harach *et al* [89]	11	Argentina	Patients with CS have increased likelihood of developing benign thyroid lesions, with increased risk of malignant transformation
Ngeow *et al* [90]	2723	United States	CS and CS-like phenotypes possess standardized incidence rate for thyroid cancer of 72%, particularly follicular thyroid cancer
Heald *et al*[91]	127	North America and Europe	*PTEN* mutations are associated with early-onset (age < 50) colorectal malignancy; routine colonoscopy should be encouraged

PTEN: *Phosphatase and tensin homolog*; CS: Cowden syndrome.

Table 5 Malignancy screening for *phosphatase and tensin homolog* mutation[92]

Malignancy	Screening
Breast	Annual self-breast exam at the age of 18; annual clinical breast exam at the age of 25; annual MRI at the age of 30
Colon	Colonoscopy every 5 yr at the age of 35, and as needed
Endometrial	Annual exam with biopsy at the age of 35, and as needed. Hysterectomy upon childbearing completion
Kidney	Annual renal ultrasound at the age of 40; CT or MRI as indicated
Skin lesion	Annual dermatologic exams, no consensus on initiation age
Thyroid	Annual thyroid ultrasound at the age of 7

MRI: Magnetic resonance imaging; CT: Computed tomography.

mutation[46]. SOLAMEN syndrome is also associated with hamartomatous polyps, macrocephaly, lipomas, and hemangiomas. These patients further possess an increased risk of skin, GI, thyroid, breast, brain, and genito-urinary malignant neoplasms[47,48]. Surveillance is advised to be similar to CS, given the mutual *PTEN* mutation association (Table 5). Treatment is predominantly symptomatic and often requires a multidisciplinary approach[49].

PS and PLS are similar pathologic processes that may be associated with a *PTEN* mutations[50]. PS has been associated with *AKT1* and *PTEN* mutations, both of which influence the PI3KCA/AKT pathway[51]. Pulmonary embolism secondary to a hypercoagulable state and pneumonia are noted to be contributing factors to early mortality in this patient population. Other complications include scoliosis, central nervous system abnormalities, ophthalmic complications, abnormal bone growth, and pulmonary malformations[52]. Treatment is often targeted toward the correction of the functional limitations caused by the skeletal deformities associated with the disease. Patients should be closely monitored for the development of venous thrombosis; however, no recommendations for prophylactic anticoagulation currently exist[3,53]. Patients that have presentations similar to PS, but do not possess

all the diagnostic criteria are deemed to have PLS.

JPS

Diagnosis

JPS is an autosomal dominant condition resulting from a germline mutation of the *SMAD4* or *BMPR1A* genes in 60% of the affected population. These genes impact the transforming growth factor-beta signaling pathway[54,55]. The estimated incidence of JPS is approximately between 1:100000 to 1:160000 [56,57]. Clinical diagnosis is based on the presence of one of the following: 5+ juvenile polyps in the colorectal region, 2+ juvenile polyps in the GI tract, or any number of juvenile polyps with a family history of juvenile polyposis[18,56,58,59]. Juvenile polyps refer to the histopathology of the polyps, rather than the age of onset. Juvenile polyps are hamartomas demonstrating a dense stroma with a smooth surface and mucin-filled cystic lamina propria[60]. Macroscopically, these pedunculated polyps have an erosive surface ranging from 5 mm to 50 mm in size. While distinction with sporadic juvenile polyps is difficult to make, JPS polyps frequently demonstrate epithelial neoplastic transformation, reduced stroma, reduced dilated glands, and increased proliferative glands[61].

Subtypes of JPS include JPS of infancy, generalized JPS, and juvenile polyposis coli. JPS of infancy is characterized by sessile or pedunculated polyps diffusely throughout the GI tract ranging from 1-30 mm. Although further investigation must be pursued, some sources have reported JPS of infancy is caused by mutations resulting in the continuous deletion of *BMPR1A* and *PTEN*[62]. The disease process is plagued with early mortality secondary to hemorrhage, malnutrition caused by diarrhea, and intussusception[61,63]. Considered to be a variable spectrum of penetrance of the same disease, generalized JPS occurs diffusely throughout the GI tract while juvenile polyposis coli is limited to the colorectal region[64]. Nearly half of the patients with generalized JPS or juvenile polyposis coli possess a heterozygous germline mutation of the *SMAD4* or *BMPR1A* gene[64].

Extra-intestinal manifestations are present in up to 15% of patients including midgut rotation, cardiac abnormalities, and craniofacial abnormalities. JPS may also be present in conjunction to hereditary hemorrhagic telangiectasia (HHT) syndrome[65,66]. Patients with HHT have a characteristic increased risk of bleeding secondary to greater rates of arteriovenous malformations of the lung, liver, brain, and GI tract. HHS is an autosomal dominant disorder also associated with a *SMAD4* gene mutation[65,67,68]. Patient may demonstrate varying degrees of disease qualities when they have both disorders simultaneously. However, the combination of both disorders results in a higher risk of colorectal malignant neoplasms and anemia[68,69]. Although further studies are required, the current perspective on the malignant pathogenesis of JPS includes the "landscaper mechanism" and the "gatekeeper" model. The "landscaper mechanism" postulates that genetic alterations lead to an abnormal stromal environment, which leads to malignant transformation of neighboring epithelium. A de novo study by Haramis *et al*[70] demonstrated that bone morphogenetic protein (*BMP*)-4 inhibition, a common JPS pathway mutation, results in ectopic crypt units adjacent to the crypt-villus axis supporting the "landscaper mechanism"[70]. A "gatekeeper" model has also been proposed, where the biallelic *SMAD4* mutation contributes to malignant transformation and progression[71,72].

Surveillance

JPS has an established increased risk of GI malignancy, with a cumulative lifetime risk reported to range between 39%-63%[73,74]. The *SMAD4* gene has been associated with a greater risk of GI malignancies as well as a greater number of polyps in comparison to the *BMPR1A* mutation[54,75,76]. JPS is associated with an elevated risk of colorectal and gastric malignancies (Table 6). Therefore, a combination of colonoscopy and EGD is recommended for appropriate screening and surveillance[77] (Table 7).

Management

Endoscopic management is typically feasible for patients with a limited number of polyps[18]. EGD may be utilized for surveillance and endoscopic removal of polyps located in the stomach and duodenum. In the setting of extensive polyposis, advanced dysplasia, or malignancy, a partial or complete gastrectomy may be pursued[44,78] Jaoude *et al*[55] recommended proposed prophylactic gastrectomy in patients with symptomatic JPS, asymptomatic patients with a *SMAD4* mutation, or patients with unmanageable polyposis defined as > 50-100 polyps[55]. Certainly, morbidity and mortality are greater with surgical intervention. However, surgical intervention must be weighed against the possibility of inadequate surveillance and treatment with endoscopy, particularly in the setting of an elevated likelihood for sporadic gastric malignancy associated with the *SMAD4* gene mutation. Further investigation should be pursued to determine the optimal parameters for surgical intervention, particularly in the prophylactic setting.

Small bowel polyps should be evaluated periodically with enteroscopy. Patient with limited small bowel polyps may be managed with endoscopic polypectomy but will require surgical treatment in the event of obstructive symptoms associated with intussusception. Colonoscopy is an essential screening and management tool for colonic polyps. However, in the setting of numerous polyps, advanced

Table 6 Studies investing the malignancy risk associated with juvenile polyposis syndrome

Ref.	N	Location	Outcome
Brosens *et al*[73]	84	United States	Patients with JPS possess a lifetime cumulative risk of 38.7% for development of colorectal cancer, with the average age of diagnosis being 43.9
Latchford *et al*[56]	44	United Kingdom	The *SMAD4* gene is a poor prognostic indicator for development of gastric malignancy. Colonic polyps have predominance to ascending colon
Howe *et al*[93]	117	United States	Kindred of patients with JPS possess approximately 50% cumulative risk of developing gastric malignancy

JPS: Juvenile polyposis syndrome.

Table 7 Malignancy screening for juvenile polyposis syndrome[18,77,94-96]

Malignancy	Screening
Colorectal	Colonoscopy every 1-3 yr between the ages of 12-15; screening should be initiated sooner if earlier onset of symptoms
Gastric	Esophagogastroduodenoscopy every 1-3 yr between the age of 12-15; screening should be initiated sooner if earlier onset of symptoms
HHT mutation	Brain MRI, cardiac echocardiogram, testing for lung arterio-venous malformations

HHT: Hereditary hemorrhagic telangiectasia; MRI: Magnetic resonance imaging.

dysplasia, or malignancy, a colectomy may be pursued. Surgical options include proctocolectomy with ileal pouch anal anastomosis (IPAA) or colectomy with ileorectal anastomosis (IRA) based on rectal involvement. Nearly half of patients who elect to undergo IRA will require additional proctocolectomy with IPAA secondary to the development of rectal polyposis[18,44,78].

An area of potential emerging investigation includes the utility of chemoprevention in patients with JPS. Van Hattem *et al*[79] determined an increased expression of cyclooxygenase-2, particularly in patients with a *BMPR1A* gene mutation. Further investigation should be pursued in order to determine the clinical implications of this expression profile[79]. Ultimately, additional studies should be pursued regarding the optimal management of these patients.

CONCLUSION

Hamartomas polyps result from hyperproliferations of normal tissues and are often associated with genetic mutations. While patients experience symptoms from the hamartomas themselves, there also exists an established malignancy risk associated with these disorders. Therefore, patient education regarding surveillance is imperative, and a multidisciplinary approach is often required for comprehensive management. First degree relatives should also be investigated for the diagnosis due to the dominant inheritance patterns that these disease processes possess. Due to the rarity of the disorders, limited studies are available on the clinical and molecular aspects of the disease processes, and further investigation should be dedicated to understanding the pathology associated with these syndromes.

FOOTNOTES

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Country/Territory of origin: United States

ORCID number: Leva Gorji 0000-0001-6837-5317.

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ORIGINAL ARTICLE

Basic Study

Stress granules inhibit endoplasmic reticulum stress-mediated apoptosis during hypoxia-induced injury in acute liver failure

Wen-Yuan Li, Fan Yang, Xun Li, Lu-Wen Wang, Yao Wang

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Wen-Yuan Li, Department of Anesthesiology, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China

Fan Yang, Xun Li, Lu-Wen Wang, Yao Wang, Department of Infectious Diseases, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China

Corresponding author: Yao Wang, Doctor, Chief Physician, Department of Infectious Diseases, Renmin Hospital of Wuhan University, No. 238 Jiefang Road, Wuhan 430060, Hubei Province, China. rm003743@whu.edu.cn

Abstract

BACKGROUND

Stress granules (SGs) could be formed under different stimulation to inhibit cell injury.

AIM

To investigate whether SGs could protect hepatocytes from hypoxia-induced damage during acute liver failure (ALF) by reducing endoplasmic reticulum stress (ERS) mediated apoptosis.

METHODS

The agonist of SGs, arsenite (Ars) was used to intervene hypoxia-induced hepatocyte injury cellular model and ALF mice models. Further, the siRNA of activating transcription factor 4 (ATF4) and SGs inhibitor anisomycin was then used to intervene in cell models.

RESULTS

With the increase of hypoxia time from 4 h to 12 h, the levels of HIF-1α, ERS and apoptosis gradually increased, and the expression of SGs marker G3BP1 and TIA-1 was increased and then decreased. Compared with the hypoxia cell model group and ALF mice model, the levels of HIF-1α, apoptosis and ERS were increased in the Ars intervention group. After siRNA-ATF4 intervention, the level of SGs in cells increased, and the levels of HIF-1α, ERS and apoptosis decreased. Compared with the siRNA-ATF4 group, the levels of G3BP1 in the siRNA-ATF4+anisomycin group were decreased, and the levels of HIF-1α, ERS and apoptosis were increased. Moreover, compared with the ALF group, the degree of liver injury and liver function, the levels of HIF-1α, ERS and apoptosis in the Ars intervention group were decreased, the level of SGs was increased.

CONCLUSION
SGs could protect hepatocytes from hypoxia-induced damage during ALF by reducing ERS-mediated apoptosis.

Key Words: Acute liver failure; Stress granules; Hypoxia; Endoplasmic reticulum stress; Apoptosis

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Core Tip: Hepatocytes were damaged by hypoxia and ischemia injury in the process of acute liver failure. At this time, the content of HIF-1α in cells increased, which inhibited the formation of stress granules (SGs) mediated by G3BP1 and promoted the expression of endoplasmic reticulum stress (ERS) marker molecules activating transcription factor 4 (ATF4) and CCAAT/enhancer-binding protein-homologous protein. The activated ERS pathway further promotes hepatocyte apoptosis. Promoting SGs synthesis can inhibit the level of hepatocyte apoptosis by inhibiting the ATF4-mediated ERS pathway.

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INTRODUCTION

Acute liver failure (ALF) is a common clinical syndrome in intensive care units, with a high mortality due to various causes, such as drugs, toxic exposure, viral hepatitis, *etc*[1]. ALF can be preliminarily diagnosed in patients with no history of basic liver disease, with rapid deepening of jaundice in a short period of time. It has the typical symptom, such as poor appetite, vomiting, diarrhea, abdominal distension and hepatic encephalopathy. Advanced high bilirubin or aminotransferase, and prothrombin activity of coagulation item ≤ 40% can be the basic diagnosis[2]. For such patients, it is extremely important to be transferred to intensive care unit as soon as possible for mechanical ventilation to improve oxygenation. The comprehensive medical treatment, blood purification (artificial liver) and maintenance of homeostasis in human body should be carried out throughout the treatment. For patients with ALF, liver transplantation is the only completely effective treatment option. However, the development of liver transplantation is limited by shortage of liver source, strict transplantation conditions, huge treatment cost and rejection after transplantation[3].

Endoplasmic reticulum stress (ERS) plays an important role in the regulation of inflammatory response and apoptosis, and severe ERS promotes the occurrence and development of ALF[4]. When cells undergo ERS, they can be induced by type-1 ER transmembrane protein kinase (IRE1), double-stranded RNA-dependent protein kinase-like ER kinase (PERK) and activating transcription factor 6 (ATF6), induce unfolded protein response (UPR)[5]. Unfolded proteins can alter cellular transcription and translation programs to regulate protein synthesis, secretion, and degradation to relieve endoplasmic reticulum stress, thereby protecting cells[6]. When ERS exceeds the controllable range of the body, cells will undergo apoptosis. CCAAT/enhancer-binding protein-homologous protein (CHOP) is a transcription factor that controls gene-encoded components in apoptosis[7]. The activation of ERS of the three pathways will eventually induce the expression of the apoptotic factor CHOP[7]. Among them, PERK can activate eIF2a and further induce the expression of ATF4. ATF4 can promote the activation of CHOP, and the highly expressed CHOP triggers the bax/bad system to activate caspase9 and caspase3, which in turn leads to apoptosis[8].

Stress granules (SGs) refer to the fact that when eukaryotic cells are subjected to various environmental stimuli, such as oxidative stress, heat shock, ultraviolet radiation, and viral infection. Aids in the transport of dense granular material formed into the cytoplasm, which is an adaptive regulation mechanism of cells[9]. SGs are a protective mechanism of cells, which is the product of the host's response to external stress. The production mechanism of SGs is related to neurological diseases[10], viral infections[11], and cancers[12], *etc*. There is no study on the relationship between SGs, ERS and hepatocyte apoptosis in ALF. This experiment investigates the effect and mechanism of SGs on the apoptosis process induced by ERS during the hypoxic injury of hepatocytes in ALF.

MATERIALS AND METHODS

Chemicals and reagents

The normal human liver L02 cell line was purchased from the China Center for Type Culture Collection (Wuhan University). DMEM medium and fetal bovine serum (FBS) were purchased from GIBCO (NY, United States). Lipopolysaccharide (LPS), D-galactosamine (D-Gal), arsenite (Ars) and anisomycin were purchased from Sigma (St. Louis, United States). Rabbit anti-human/ mouse G3BP1, T-cell restricted intracellular antigen-1 (TIA-1), ATF4, CHOP, BAX, BCL2, GAPDH antibodies, Cy3-labeled goat anti-rabbit secondary antibody were purchased from Proteintech (Wuhan, China). Rabbit anti-human/ mouse cleaved caspase3 (c-cas3), c-cas9 antibodies were purchased from Cell Signaling Technology (Boston, United States). Goat anti-rabbit fluorescent secondary antibody IRDye800 was purchased from LI-COR Biosciences (Lincoln, United States). Human and mouse lactate dehydrogenase (LDH) and HIF-1α enzyme-linked immunosorbent assay kits were purchased from Elabscience (Wuhan, China). RNAiso Plus, PrimeScript™, RT reagent and SYBR Premix Ex Taq kit were purchased from TaKaRa (Dalian, China). The Annexin V-phycoerythrin/7-aminoactinomycin D (Annexin V-PE/7AAD) apoptosis kit Apoptosis kit was purchased from BD (San Diego, United States). The transfection reagent Lipo200 was purchased from Invitrogen (CA, United States).

Cell culture and treatment

After receiving the L02 cells, they were centrifuged to remove the cell cryopreservation solution. The cells were then added DMEM complete medium (containing 10% FBS), and cultured in a 37 °C 5% CO_2 constant temperature incubator in a suitable humidity environment. The adherence and growth of the cells were observed under a microscope, and the cells were in the logarithmic growth phase for experiments. The hypoxia-treated cell group was placed in a three-gas incubator at 37 °C with a volume fraction of 95% N_2 + a volume fraction of 5% CO_2 for hypoxia treatment for 4 h, 8 h, and 12 h, respectively. Hepatocytes treated with hypoxia for 12 h were used as model group. The Hypoxia + Ars group was first treated with Hypoxia for 12 h, followed by hypoxia treatment for 12 h. Hypoxia + anisomycin + siRNA-ATF4 group cells were first transfected with siRNA, then intervened with anisomycin for 12 h, and finally treated with hypoxia for 12 h. The sequence of siRNA-ATF4 was 5'-TCC CTC AGT GCA TAA AGG A-3'. And the sequence of non-targeting control siRNA was 5'-UUC UCC GAA CGU GUC ACG A-3'. The siRNA was transfected into cells using Lipo2000 according to the manufacturer's instructions.

Animal groups and treatment

All animal experimental procedures were evaluated and approved by the Laboratory Animal Care and Committee of Renmin Hospital of Wuhan University (WDRM20181018) and followed institutional guidelines as well as ARRIVE guidelines. Thirty male C57BL/6 rats were randomly divided into 3 groups, namely the normal group, the model group, and the Ars group, with 10 rats in each group. All mice were acclimated to feeding for 1 wk before the experiment. Referring to the previous modeling method[13,14], the mice in the model group and Ars group were injected with D-Gal 400 mg/kg and LPS 100 μg/kg intraperitoneally. The mice in Ars group were given Ars (1 mg/kg) intraperitoneal treatment 3 d before modeling, once a day, until 24 h after modeling. The rats in the normal group and the model group were given normal saline by gavage every day. 12 h after modeling, the survival of mice in each group was observed and recorded. Body weights and health of the animals were monitored every other day. If the mice were unable to eat or drink, showed any abnormal behavior, or signs of toxicity, pain, or distress, they would be removed from the study. At the end of the experiment, the mice were euthanized by sodium pentobarbital, and death was confirmed by the absence of a heartbeat. About 1 mL of ocular venous blood was collected. Liver tissue was taken, washed with normal saline, and then stored in -80 °C freezer for tissue homogenate, protein and mRNA extraction. The rest of the liver tissue specimens were fixed with 4% paraformaldehyde, embedded in paraffin, and used for hematoxylin-eosin (HE) and transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) staining.

Cell apoptosis, HIF-1α and LDH detection

Referring to the previous method[15], the apoptosis level of L02 cells in each group was detected by flow cytometry using Annexin V-PE/7AAD apoptosis kit. After intervention in each group, 1.0×10^5 cells were added to 400 μL buffer solution for staining. 5 μL of Annexin V-PE and 5 μL of 7AAD were then added to the cell suspension. The cell was incubated in the dark for 15 min at 37 °C. The apoptosis rate of early and late cells was detected by flow cytometry (BD, United States). According to the description in the previous experiments[16], the cell supernatant, homogenates of cells and liver tissues in each group were taken. Then the HIF-1α and LDH detection kits were used to evaluate the content of HIF-1α in cell or tissue homogenates, LDH in cell supernatant or serum according to the kit instructions.

Determination of G3BP1 expression by immunofluorescence

The cells in each group were modeled and intervened by the above method. After 24 h of incubation, they were fixed with cell fixative for 25 min, and then washed with PBS for 3 times. 0.2% Triton was permeabilized for 20 min, and 0.5% BSA was blocked for 30 min. G3BP1 primary antibody (1:1000) was incubated overnight. The next day, the secondary antibody was incubated for 1 h, nuclei were stained with DAPI for 3 min, washed with PBS for 5 times. The anti-fluorescence quencher was added dropwise, and the slides were mounted and observed under an upright microscope.

Detection of ATF4 mRNA by quantitative real-time polymerase chain reaction

The total RNA of each group of cells was extracted by RNAiso Plus kit. The cDNA was further amplified using Prime-Script RT reagent kit. The amplified cDNA was subjected to quantitative polymerase chain reaction (qPCR) by real-time PCR (RT-PCR) using SYBR Premix Ex Taq kit. The PCR program was set as: initial 95 °C for 10 s; 95 °C for 5 s and 60 °C for 20 s, 40 cycles. ATF4 primer sequences: forward 5'-TCAAACCTCATGGGTTCTCC-3' and reverse 5'-GTGTCATCCAACGT-GGTCAG-3'. GAPDH primer sequences: forward 5'-ACCACAGTCCATGCCATCAC-3', reverse 5'-TCCACCACCCTGTTGCTGTA-3'. The relative gene expression of ATF4 was calculated by $2^{-\Delta\Delta CT}$. The above methods refer to previous reports[17].

Protein expression was detected by western blot

Referring to the previous experimental method[18], the cells or liver tissues of each group were collected into the centrifuge tube, and were broken under ice bath by ultrasound. After centrifugation at 4 °C, the supernatant was taken. Total protein was determined by bicinchoninic acid method and protein concentration was adjusted. It was then added protein loading buffer, mixed well, and boiled for 10 min. Electrophoresis was performed with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. 10 μL sample to each well was then add. Electrophoresis was performed at 80 V and then at a constant current of 200 mA. PVDF membranes were sealed with 5% skim milk powder at room temperature for 1h. Then primary antibody (G3BP1, 1:1,000; TIA-1, 1:1000; ATF4, 1:1000; CHOP, 1:1000; BAX, 1:1000; BCL2, 1:1000; c-cas3, 1:1,000; c-cas9, 1:1000; GAPDH, 1:3000) was used to incubated the membranes overnight at 4 °C. After the membranes were washed, fluorescent secondary antibody (1:10000) was used to incubate at room temperature without light for 1 h. Finally, the membranes were scanned with Odyssey system to detect and compare the relative protein expression levels of each group.

Biochemical tests and histological studies

The levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBIL) in serum in each group were detected by automatic biochemical analyzer. Liver tissues fixed by formaldehyde and embedded in paraffin were taken from each group. The paraffin blocks were sectioned. Hematoxylin and eosin were used to stain the slices. The slices were dehydrated and dried in turn. The histopathological changes of liver in each group were observed under inverted microscope. The slices were stained with TUNEL and incubated away from light. After washing with PBS, DAPI was dropped and incubated away from light. Cell morphology was observed under fluorescence microscope, and apoptosis index in each tissue was calculated according to the method described previously[19].

Statistical analysis

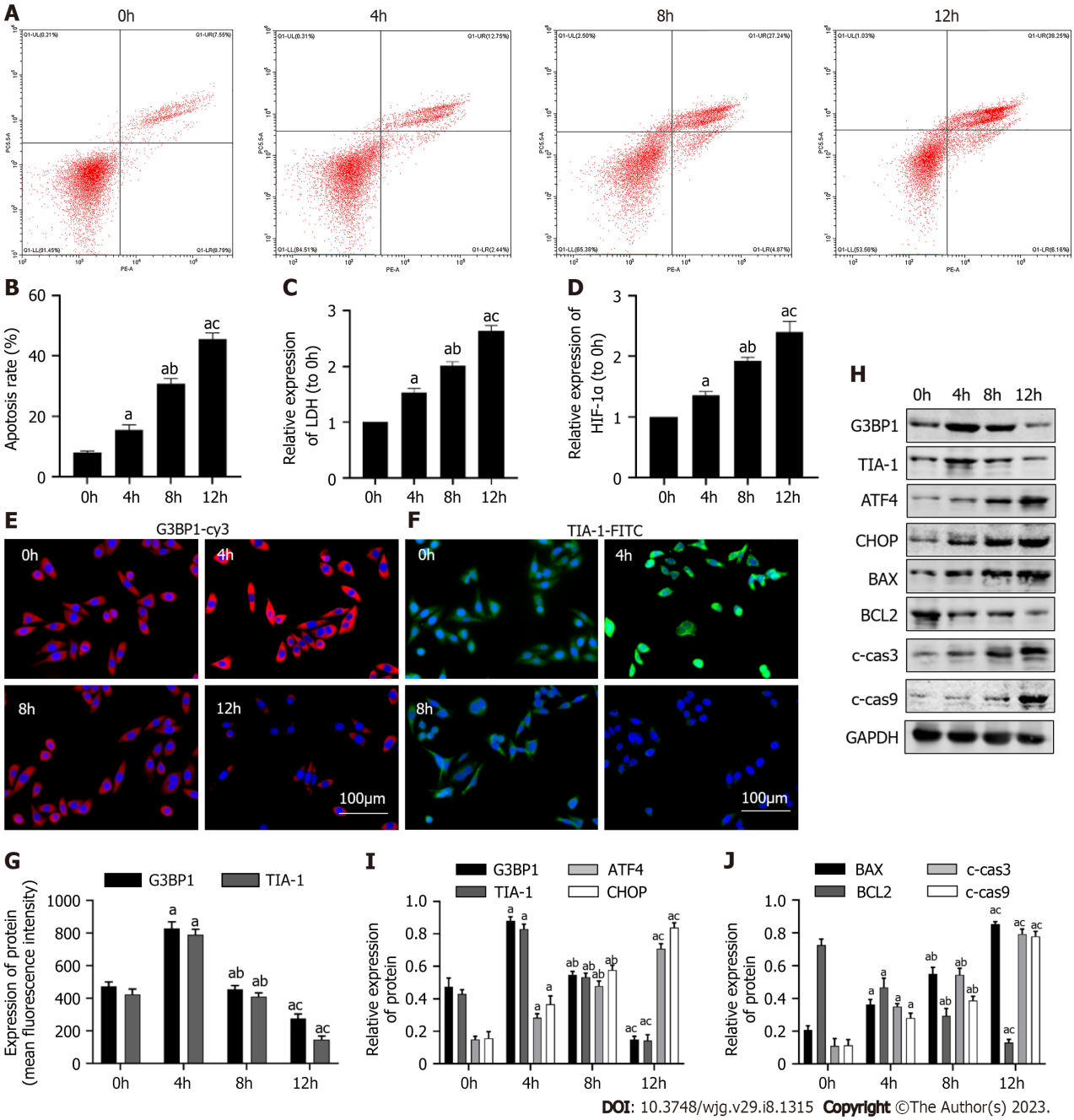
SPSS 19.0 statistical software was used for statistical analysis. All data were expressed as mean ± SD. *t*-test was used for comparison between two groups, and analysis of variance test was used for comparison between three groups and more than three groups. $P < 0.05$ means the difference was statistically significant.

RESULTS

Changes of SGs, ERS and apoptosis levels with the change of hypoxia time

The histopathological examination of liver failure revealed massive necrosis and extensive intrahepatic inflammatory reaction, resulting in the embolization of micro vessels and the structural destruction of hepatic sinuses. The microcirculation disturbance could lead to obvious hepatic perfusion obstruction, reflecting ischemic hypoxic injury[20]. The ischemia-hypoxia and ischemia-reperfusion (IR) could further activate local inflammatory response or aggravate local perfusion disorders through platelet activation pathway[21].

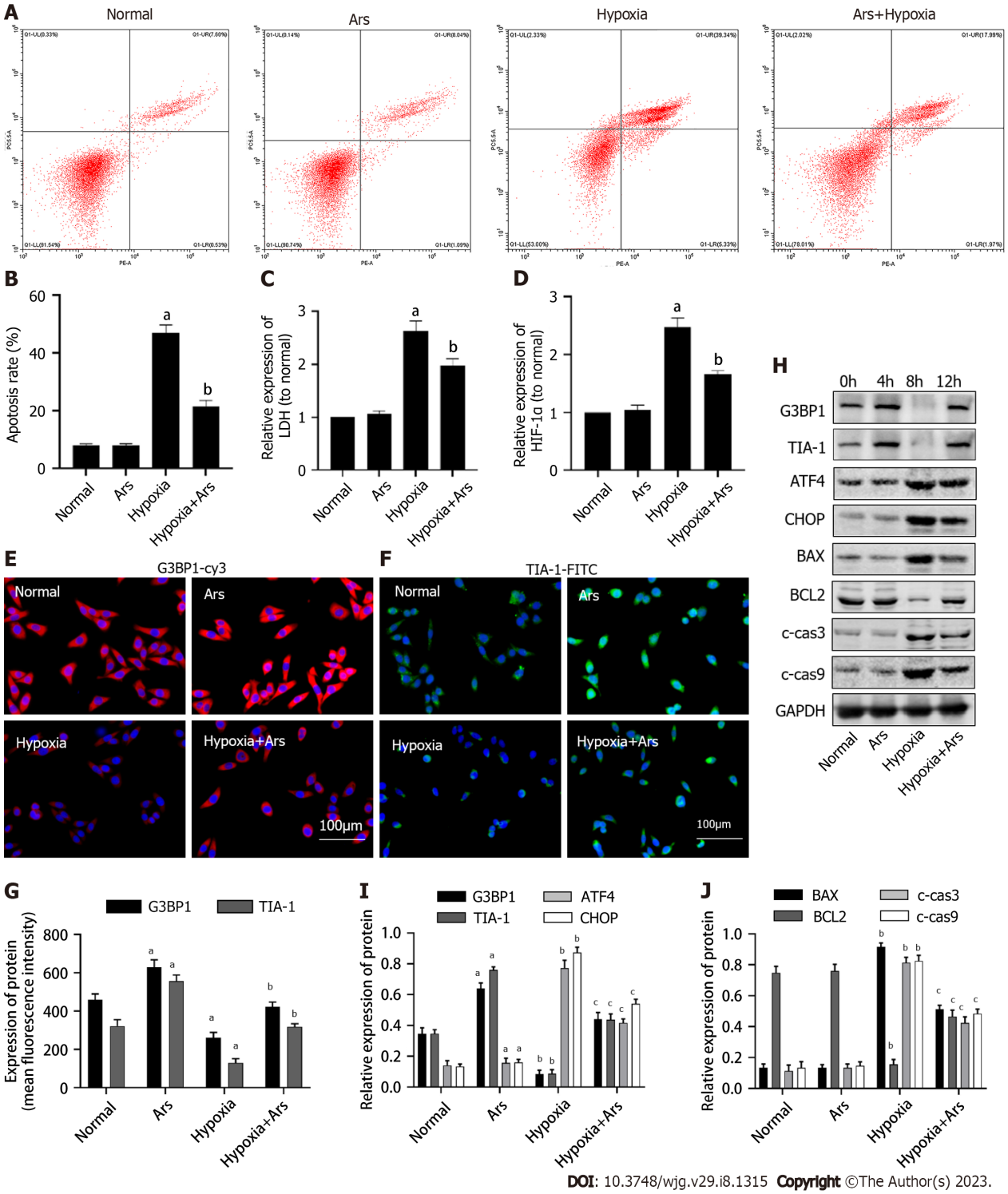
In this part, the model of hepatocyte hypoxia *in vitro* was used to simulate the ischemic hypoxia injury during ALF. It was firstly observed how the levels of SGs, ERS and apoptosis changed over time. As shown in Figure 1A-D, compared with the normal group, the apoptosis rate, HIF-1α and LDH contents in the hypoxia group for 4 h were increased ($P < 0.05$). Compared with the 4 h group, the apoptosis rate and the contents of HIF-1α and LDH in hypoxia group for 8 h and 12 h was furtherly



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Figure 1 Changes of stress granules, endoplasmic reticulum stress and apoptosis levels with the change of hypoxia time. A and B: The level of apoptosis was detected by flow cytometry; C and D: The detection kits were used to evaluate the content of HIF-1α in homogenates, lactate dehydrogenase in cell supernatant; E: The expression of G3BP1 in cells was detected by immunofluorescence; F: The expression of TIA-1 in cells was detected by immunofluorescence; G: The histogram displays the amount of molecules expression; H-J: The protein expression levels of G3BP1, TIA-1, ATF4, CHOP, BAX, BCL2, c-cas3 and c-cas9 was detected by western blot. $n = 3$. Data was shown mean ± SD. $^{a}P < 0.05$ *vs* 0 h group; $^{b}P < 0.05$ *vs* 4 h group; $^{c}P < 0.05$ *vs* 8 h group.

increased ($P < 0.05$). As shown in Figure 1E-G, compared with the normal group, the expression levels of G3BP1 and TIA-1, the marker molecules of SGs, were increased in the hypoxia group for 4 h ($P < 0.05$). The expression of G3BP1 decreased in the hypoxia groups of 8 h and 12 h compared with the hypoxia group of 4 h ($P < 0.05$). As shown in Figure 1H-J, compared with the normal group, the protein expression levels of ATF4, CHOP, BAX, c-cas3 and c-cas9 in the hypoxia group for 4 h increased ($P < 0.05$), the expression of G3BP1, TIA-1, BCL2 was decreased ($P < 0.05$). After 8 h and 12 h hypoxia, the protein expression levels of ATF4, CHOP, BAX, c-cas3 and c-cas9 were further increased ($P < 0.05$), the expression of G3BP1, TIA-1, BCL2 was further decreased ($P < 0.05$). In conclusion, after 12 h hypoxia treatment, ERS and apoptosis levels of L02 cells were the highest, while SGs content was the lowest. Therefore, the cells treated with hypoxia for 12 h were used as cell models for subsequent research, which was consistent with reported studies[22].



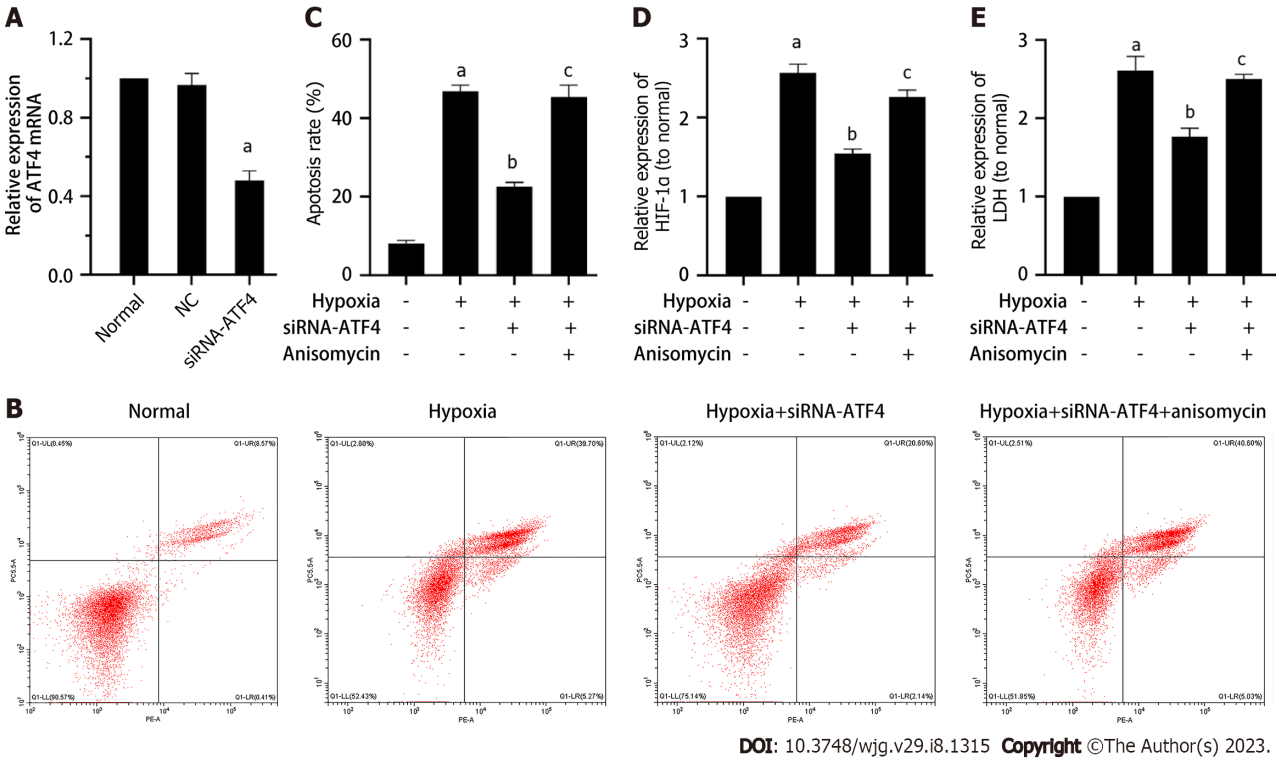
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Figure 2 Effects of stress granules agonist on endoplasmic reticulum stress and apoptosis in hepatocyte hypoxia model. A and B: The level of apoptosis was detected by flow cytometry; C and D: The detection kits were used to evaluate the content of HIF-1α in homogenates, lactate dehydrogenase in cell supernatant; E: The expression of G3BP1 in cells was detected by immunofluorescence; F: The expression of TIA-1 in cells was detected by immunofluorescence; G: The histogram displays the amount of molecules expression; H-J: The protein expression levels of G3BP1, TIA-1, ATF4, CHOP, BAX, BCL2, c-cas3 and c-cas9 was detected by western blot. $n = 3$. Data was shown mean ± SD. $^{a}P < 0.05$ *vs* normal group; $^{b}P < 0.05$ *vs* hypoxia group.

Effects of SGs on ERS and apoptosis in hepatocyte hypoxia model

Arsenite can induce SGs formation by promoting e-IF2α phosphorylation[23], but arsenite has certain hepatotoxicity[24,25]. Refer to the reported studies, the 10 μmol/L intervention dose was used[25,26]. In this part, the Ars intervention group was set to observe that Ars could induce the formation of SGs without causing hepatocyte damage and related molecular changes.

As shown in Figure 2, we first verified the effect of SGs agonist Ars on normal L02 cells. Compared with the normal group, the expression level of G3BP1 was increased in the Ars group, while the level of



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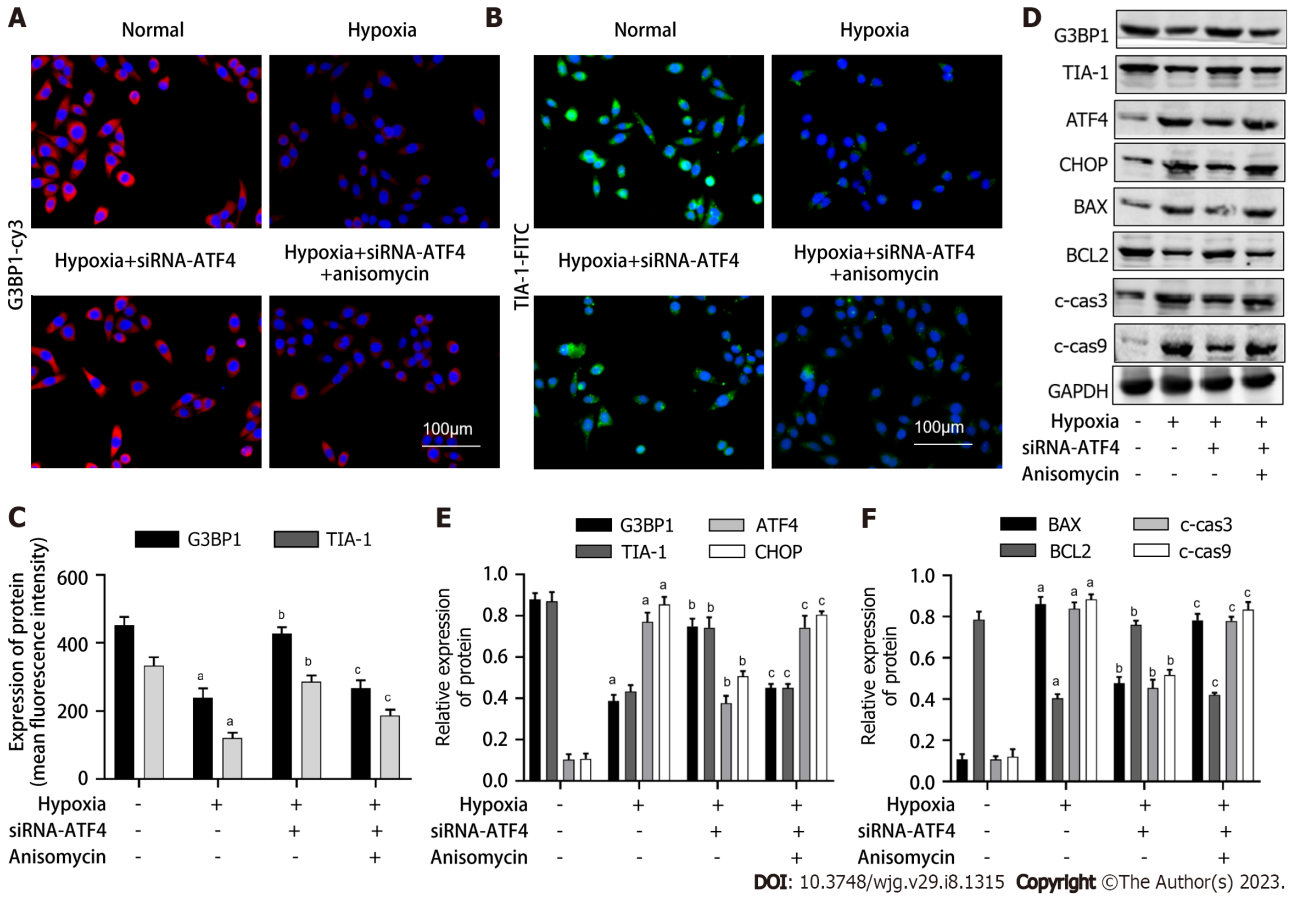
Figure 3 Effect of stress granules on apoptosis, HIF-1α, and lactate dehydrogenase in hepatocyte hypoxia model through endoplasmic reticulum stress. A: The level of mRNA in cells was detected by quantitative real-time polymerase chain reaction; B and C: The level of apoptosis was detected by flow cytometry; D-E: The detection kits were used to evaluate the content of HIF-1α in homogenates, lactate dehydrogenase in cell supernatant. n = 3. Data was shown mean ± SD. $^{a}P < 0.05$ *vs* normal group; $^{b}P < 0.05$ *vs* hypoxia group; $^{c}P < 0.05$ *vs* hypoxia + siRNA-ATF4.

ERS and apoptosis did not change significantly. Subsequently, we used Ars to intervene the L02 hepatocyte model treated with 12 h hypoxia. As shown in Figure 2A-D, compared with the Hypoxia group, the apoptosis rate, HIF-1α and LDH contents in cells in the Hypoxia + Ars group were decreased ($P < 0.05$). As shown in Figure 2E-G, compared with the Hypoxia group, the expression levels of G3BP1 and TIA-1 were increased in the Hypoxia + Ars group ($P < 0.05$). As shown in Figure 2H-J, compared with the Hypoxia group, the protein expression levels of ATF4, CHOP, BAX, c-cas3 and c-cas9 in the Hypoxia + Ars group was decreased ($P < 0.05$), the expression of G3BP1, TIA-1, BCL2 was increased ($P < 0.05$).

Effect of SGs on apoptosis in hypoxia model through ERS

ATF4 plays a key role in the process of ERS, which negatively regulates Bcl-2 by promoting the expression of CHOP, leading to the transport of Bax from cytoplasm to mitochondria and the initiation of mitochondrial apoptosis pathway[27]. When excessive ERS occurs, PERK, IRE1, and ATF4 activate and activate the corresponding downstream factors, and up-regulate the expression of ATF4 at the transcriptional and translational levels, resulting in the destruction of the endoplasmic reticulum membrane, the efflux of Ca^{2+}, and the occurrence of apoptosis[28]. The knockdown of ATF4 and the SGs inhibitor anisomycin showed an antagonistic effect on the protective cells. In this part, the groups were set to show that SGs can affect hepatocyte injury through the ATF4-mediated ERS pathway.

We first verified the intervention effect of siRNA-ATF4. As shown in Figure 3A, compared with the normal group, the level of ATF4 mRNA in the normal control group did not change, while the level of ATF4 mRNA in the siRNA-ATF4 group decreased significantly ($P < 0.05$). As shown in Figure 3B-E, compared with the Hypoxia group, the apoptosis rate, HIF-1α and LDH contents in cells of the Hypoxia + siRNA-ATF4 group were decreased ($P < 0.05$). Compared with the Hypoxia + siRNA-ATF4, the apoptosis rate, HIF-1 α and LDH contents in cells of the Hypoxia + siRNA-ATF4 + anisomycin group were increased ($P < 0.05$). As shown in Figure 4A-C, compared with the Hypoxia group, the expression of G3BP1 and TIA-1 in Hypoxia + siRNA-ATF4 group was increased ($P < 0.05$). Compared with the Hypoxia + siRNA-ATF4, the expression of G3BP1 and TIA-1 in Hypoxia + siRNA-ATF4 + anisomycin group was decreased ($P < 0.05$). As shown in Figure 4C-F, compared with the Hypoxia group, the protein expression levels of ATF4, CHOP, BAX, c-cas3 and c-cas9 in the Hypoxia + siRNA-ATF4 group was decreased ($P < 0.05$), the expression of G3BP1, TIA-1, BCL2 was increased ($P < 0.05$). Compared with the Hypoxia + siRNA-ATF4 group, the protein expression levels of ATF4, CHOP, BAX, c-cas3 and c-cas9 in the Hypoxia + siRNA-ATF4 + anisomycin group was increased ($P < 0.05$), the expression of



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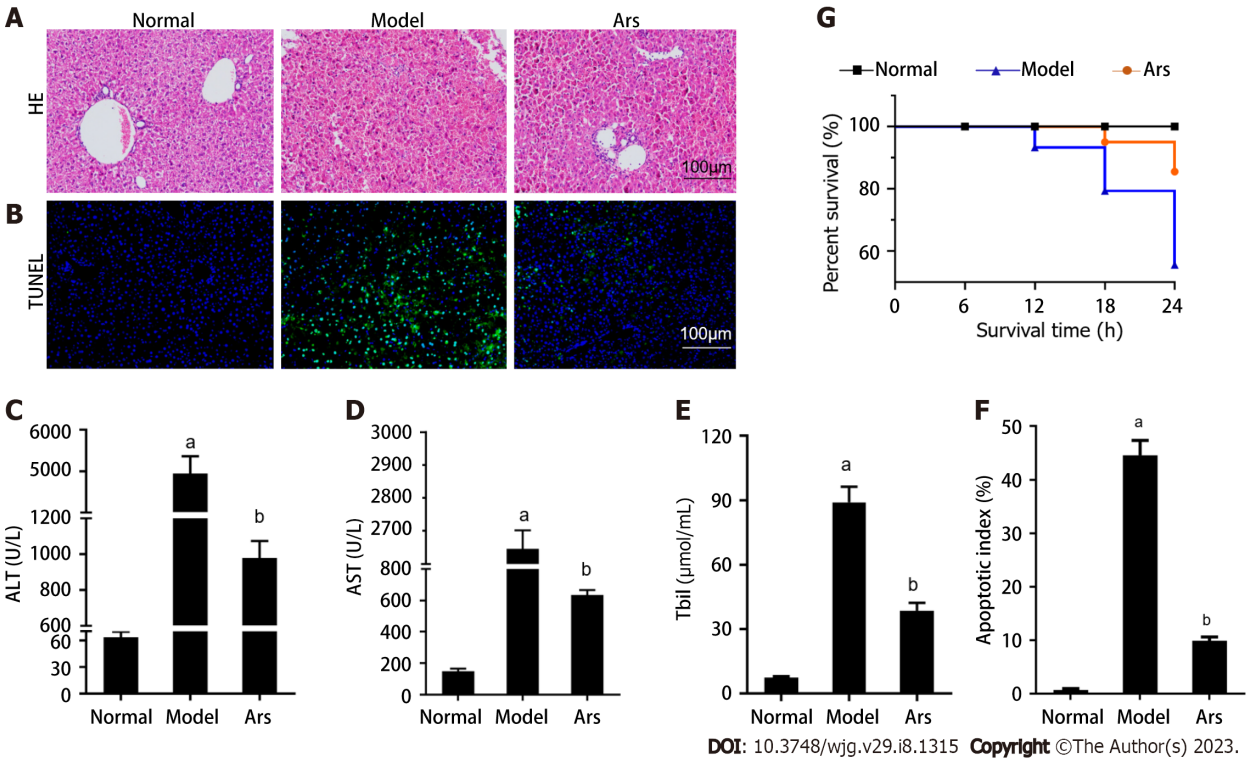
Figure 4 Effect of stress granules on G3BP1 and endoplasmic reticulum stress related molecules in hepatocyte hypoxia model through endoplasmic reticulum stress. A: The expression of G3BP1 in cells was detected by immunofluorescence; B: The expression of TIA-1 in cells was detected by immunofluorescence; C: The histogram displays the amount of molecules expression; D-F: The protein expression levels of G3BP1, TIA-1, ATF4, CHOP, BAX, BCL2, c-cas3 and c-cas9 was detected by western blot. $n = 3$. $^{a}P < 0.05$ *vs* normal group; $^{b}P < 0.05$ *vs* hypoxia group; $^{c}P < 0.05$ *vs* hypoxia + siRNA-ATF4.

BCL2 was decreased ($P < 0.05$).

Effects of SGs on ERS and apoptosis levels in liver of ALF mice

In the *in vivo* experiment, we first detected whether the ALF mouse model was successfully established, and then detected the effects of Ars on liver pathological changes and serum biochemical indexes. As shown in Figure 5A, the liver lobules in the normal group were clearly structured and the hepatocytes were neatly arranged. The lobule structure of liver tissue in ALF group was not clear, hepatocyte was necrotic around with inflammatory cells infiltrate. Compared with ALF model group, the hepatic lobule structure in Ars group was clearer and the infiltration of inflammatory cells was reduced. As shown in Figure 5B and F, compared with the normal group, the apoptosis level in liver tissues of mice in model group was significantly increased ($P < 0.05$). After Ars intervention, apoptosis level of liver tissue was significantly reduced ($P < 0.05$). As shown in Figure 5C-E, the serum levels of ALT, AST and TBIL in model group were higher than those in normal group ($P < 0.05$). Compared with model group, ALT, AST and TBIL levels in Ars group were significantly decreased ($P < 0.05$). In addition, as shown in Figure 5G, the 24 h survival rate of mice was observed in each group. The results showed that 90% of mice survived in Ars group, whereas only 70.0% in model group.

As shown in Figure 6A and B, compared with the normal group, the expression of G3BP1 and TIA-1 in model group was decreased ($P < 0.05$). Compared with the model group, the expression of G3BP1 and TIA-1 in Ars group was increased ($P < 0.05$). As shown in Figure 6C-G, compared with the normal group, the level of HIF-1α and LDH, the protein expression of ATF4, CHOP, BAX, c-cas3 and c-cas9 in the model was increased ($P < 0.05$), the expression of G3BP1, TIA-1, BCL2 was decreased ($P < 0.05$). Compared with the model group, the level of HIF-1α and LDH, the protein expression of protein expression levels of ATF4, CHOP, BAX, c-cas3 and c-cas9 in the Ars group was decreased ($P < 0.05$), the expression of BCL2 was increased ($P < 0.05$).



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Figure 5 Effects of stress granules agonist on histopathology, apoptosis levels, liver function in acute liver failure mice. A: Hepatic histopathological changes were detected by HE staining; B and F: TUNEL stanning was used to detected the level of apoptosis in live liver tissue; C-E: The levels of alanine aminotransferase, aspartate aminotransferase and total bilirubin in the serum of mice were detected by automatic biochemical analyzer; G: The 6 h, 12 h, 18 h, 24 h survival rates of mice in each group were observed. n = 10. Data was shown mean ± SD. $^{a}P < 0.05$ *vs* normal group; $^{b}P < 0.05$ *vs* model group. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin.

DISCUSSION

Liver is very sensitive to ischemic hypoxic injury because of its high aerobic tissue and metabolism. Ischemia-hypoxia and IR injury induce a large number of ERS and mitochondrial apoptosis pathway. ERS can be induced under ischemia and hypoxia through HIF-1α[29,30]. The complex functions of ERS in hepatocytes can regulate inflammation, oxidative stress, *etc*., which are closely related to the pathogenesis of liver failure. A large number of experimental studies have confirmed that ERS occurs in the liver of various types of ALF animal models. In the liver of ALF mice/rats induced by N-acetaminophen (APAP)[31], LPS combined with D-Gal[32], and carbon tetrachloride[33] induced liver, p-eIF2α, ATF4 and CHOP GRP78 markedly increased. The ERS inhibitors 4-phenylbutyric acid[34] and TUDCA[35] can alleviate the up-regulation of endoplasmic reticulum stress markers CHOP, XBP1, p-eIF2α, and reduce inflammation in liver tissue. damage, alleviate the up-regulation of serum transaminases. At the same time, inhibiting of ERS had a protective effect on APAP-induced liver injury, can reduce liver necrosis, and improve mouse survival[36]. It can be seen that ERS-related molecules can be used as new targets, and the development of drugs targeting ERS provides new ideas for the treatment of ALF.

ERS is an important homeostatic device during liver disease progression, which can assist cells to resist stress, but excessive stress can also lead to cell damage. In the early stage of stress, the endoplasmic reticulum reduces the cell load caused by the accumulation of wrong proteins by inhibiting protein synthesis and accelerating protein transport and degradation, and maintains cell stability[37]. However, when the stress intensity continues to increase, ERS will initiate cell apoptosis pathway. Among them, ATF4 induces the production of endoplasmic reticulum oxide protein and activates the inositol triphosphate receptor, which is a key way to induce apoptosis[38].

SGs are formed by RNA and protein aggregates that control RNA metabolism, signaling, and cell survival under stress. When translation is inhibited, polysomes lose their mRNAs, and "naked" mRNAs assemble with SG nucleating proteins for liquid-liquid phase separation[39]. SGs are dynamically complex and variable biomolecular condensates whose composition and structure undergo dramatic changes under different types of stress. The ability of cells to respond rapidly to various environmental stresses[40,41]. Therefore, the dynamic process of SGs and their regulation are crucial for cells to cope with stress. Studies have shown that the process of SGs formation is stalled by the accumulation of translation initiation complexes in response to various stresses[42]. G3BP1 bind mRNA and aggregate to form SGs. While large SGs aggregates are formed by smaller aggregates through post-translational

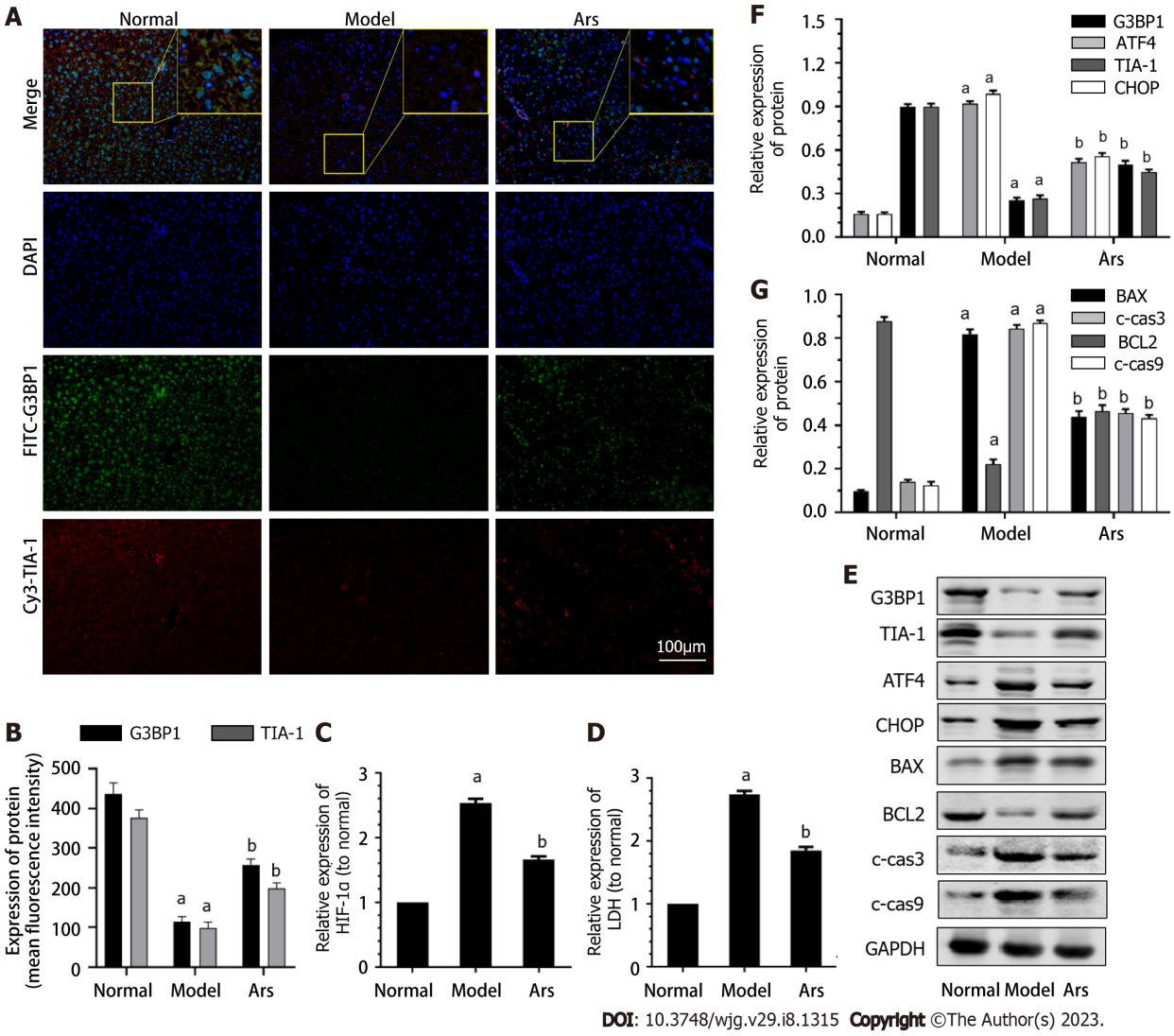


Figure 6 Effect of stress granules agonist on G3BP1, HIF-1α, lactate dehydrogenase and endoplasmic reticulum stress related molecules in acute liver failure mice liver. A: The expression of G3BP1 and TIA-1 in liver was detected by immunofluorescence; B: The histogram displays the amount of molecules expression; C and D: The detection kits were used to evaluate the content of HIF-1α in liver tissue homogenate, lactate dehydrogenase in serum; E-G: The protein expression levels of G3BP1, TIA-1, ATF4, CHOP, BAX, BCL2, c-cas3 and c-cas9 was detected by western blot. n = 10. Data was shown mean ± SD. ^{a}P < 0.05 *vs* normal group; ^{b}P < 0.05 *vs* model group.

modification and microtubule transport[43]. Another typical SGs maker, TIA-1 RNA-binding protein packet TIA-1 binds to mRNA, causing mRNA to stop translation and aggregate to form SGs[44,45]. Therefore, G3BP1 and TIA-1 were selected as markers for SGs in this study. Therefore, G3BP1 and TIA-1 were selected as markers for SGs in this study.

There is no relevant report on the relationship between SGs, ERS and hepatocyte apoptosis in the process of hepatocyte ischemia and hypoxia for ALF. In the *in vitro* study, we firstly observed how the levels of SGs, ERS and apoptosis changed over time. With the prolongation of hypoxia time, the levels of ERS and apoptosis in hepatocytes increased. The level of SGs increased at 4h and then decreased. Therefore, hepatocyte treated with hypoxia for 12 h were used as cell models for subsequent research. This suggested that SGs were stress-increased in the early stage of hepatocyte hypoxia to protect cells from damage. However, with the prolongation of hypoxia time, the production of SGs would decrease, and the effect of protecting hepatocytes will be weakened. Then the effects of SGs on ERS and apoptosis in hepatocyte hypoxia model was observed. Compared with the Hypoxia group, the apoptosis rate and ERS level decreased in SGs hepatocyte Ars treated group. However, Ars could elevate the level of SGs. In the next, it was verified the effect of SGs on apoptosis level of hepatocyte hypoxia model through ERS. It was verified the intervention effect of siRNA-ATF4. Compared with the normal group, the level of ATF4 mRNA in the siRNA-ATF4 group was decreased significantly. Compared with the Hypoxia group, the apoptosis rate, HIF-1α and LDH contents in cells, the level of ERS was decreased in the siRNA-ATF4 treatment group. Moreover, on the basis of siRNA-ATF4 intervention group, it was found that the apoptosis rate, HIF-1α and LDH contents in cells, the level of ERS was decreased.

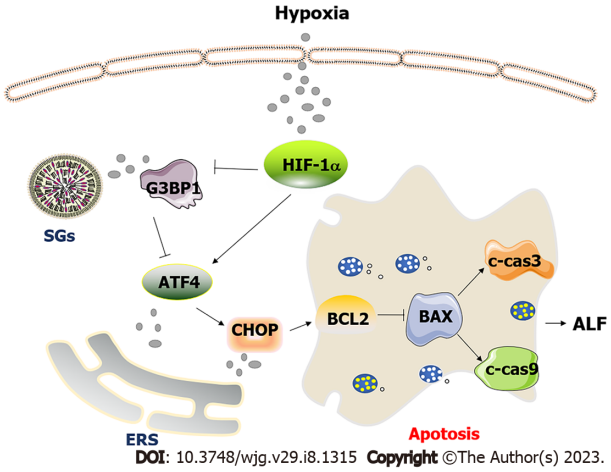


Figure 7 Graphical abstract. In the process of acute liver failure, hepatocytes were damaged by hypoxia and ischemia injury. At this time, the content of HIF-1α in cells increased, which inhibited the formation of stress granules (SGs) mediated by G3BP1 and promoted the expression of endoplasmic reticulum stress (ERS) marker molecules ATF4 and CHOP. The activated ERS pathway further promotes hepatocyte apoptosis. Promoting SGs synthesis can inhibit the level of hepatocyte apoptosis by inhibiting the ATF4-mediated ERS pathway. SGs: Stress granules; ERS: Endoplasmic reticulum stress; ALF: Acute liver failure.

According to previous studies, the criteria for success in animal modeling of ALF include liver histopathological damage, serological changes, especially elevated levels of transaminase and bilirubin [13,16,46]. In this study, LPS injection combined with D-Gal simulates acute inflammatory liver injury model, which is widely accepted and used to explore and develop new liver protective reagents for inflammatory liver injury[47]. LPS is in the outer membrane of the gram-negative bacteria[48], and can combine with myeloid differentiation factor 2 and cluster of differentiation 14 to form a complex. This complex is recognized by the toll-like receptor 4 on the membrane of Kupffer cells (KCs) in the liver. KCs can produce the inflammatory mediators, such as TNF-α, IL-1β, IL-6, *etc.*, leading to liver cell damage[49,50]. However, LPS has a low specificity for liver injury, so it is often combined with DGal to establish an animal model of acute inflammatory liver injury to simulate human hepatitis[48]. Studies have shown that D-Gal can inhibit protein synthesis by depletion of uridine triphosphate through the galactose pathway, and reactive oxygen species production can induce liver injury. Therefore, D-Gal can be used as a sensitizer in LPS-induced liver injury[51]. In the course of occurrence and development of liver failure, a large number of liver cell death is the most core event.

So, In the *in vivo* experiment, it was first detected whether the ALF mouse model was successfully established. HE staining showed that the liver tissue structure of mice in the normal group was regular, and the liver cells were neatly arranged in the hepatic lobules. No necrosis of liver cells and inflammatory cell infiltration. The liver lobule structure of the ALF model group was destroyed by LPS combined with D-Gal, accompanied by a large number of necrotic liver cells and infiltrating inflammatory cells. Compared with normal group, ALT, AST and TBIL levels in serum of model group were increased. The above studies indicate that the mouse model of ALF has been successfully constructed in this study, which is consistent with previous reports[13,16]. After Ars intervention, the degree of infiltration of necrotic liver cells and inflammatory cells in mouse liver tissue was reduced, and the levels of ALT, AST and TBIL in serum were decreased.

Moreover, compared with the normal group, the apoptosis level in liver tissues of mice in model group was significantly increased. The expression of G3BP1 in model group was decreased. The expression of G3BP1 in model group was decreased. Moreover, compared with the normal group, the level of HIF-1α and LDH, the protein expression of ATF4, CHOP, BAX, c-cas3 and c-cas9 in the model was increased, the expression of BCL2 was decreased. Compared with the model group, the level of HIF-1α and LDH, the protein expression of protein expression levels of ATF4, CHOP, BAX, c-cas3 and c-cas9 in the Ars group was decreased, the expression of BCL2 was increased.

CONCLUSION

In conclusion, as shown in Figure 7, hepatocytes were damaged by hypoxia and ischemia injury in the process of ALF. At this time, the content of HIF-1α in cells increased, which inhibited the formation of SGs mediated by G3BP1 and promoted the expression of ERS marker molecules ATF4 and CHOP. The activated ERS pathway further promotes hepatocyte apoptosis. Promoting SGs synthesis can inhibit the level of hepatocyte apoptosis by inhibiting the ATF4-mediated ERS pathway. However, the inhibition of ERS-mediated hepatocyte apoptosis pathway by SGs still needs to be further studied. Despite the use of Ars and anisomycin in the intervention of SGs, there is still no direct evidence to prove the influence of

SGs on hepatocyte apoptosis. In the next studies, we will use more appropriate methods to directly detect the influence of SGs on hepatocyte. At the same time, this paper provides potential targets and ideas for clinical treatment of ALF.

ARTICLE HIGHLIGHTS

Research background

There is no relevant report on the relationship between stress granules (SGs), endoplasmic reticulum stress (ERS) and hepatocyte apoptosis in the process of hepatocyte ischemia and hypoxia for acute liver failure (ALF).

Research motivation

This paper provides potential targets and ideas for clinical treatment of ALF.

Research objectives

This study was to investigate whether SGs could protect hepatocytes from hypoxia-induced damage during ALF by reducing ERS mediated apoptosis.

Research methods

The agonist of SGs, arsenite (Ars) was used to intervene hypoxia-induced hepatocyte injury cellular model and ALF mice models. Further, the siRNA of ATF4 and SGs inhibitor anisomycin was then used to intervene in cell models.

Research results

In the *in vitro* study, we firstly observed how the levels of SGs, ERS and apoptosis changed over time. With the prolongation of hypoxia time, the levels of ERS and apoptosis in hepatocytes increased. The level of SGs increased at 4h and then decreased. Therefore, hepatocyte treated with hypoxia for 12 h were used as cell models for subsequent research. This suggested that SGs were stress-increased in the early stage of hepatocyte hypoxia to protect cells from damage. However, with the prolongation of hypoxia time, the production of SGs would decrease, and the effect of protecting hepatocytes will be weakened. Then the effects of SGs on ERS and apoptosis in hepatocyte hypoxia model was observed. Compared with the Hypoxia group, the apoptosis rate and ERS level decreased in SGs hepatocyte Ars treated group. However, Ars could elevate the level of SGs. In the next, it was verified the effect of SGs on apoptosis level of hepatocyte hypoxia model through ERS. It was verified the intervention effect of siRNA-ATF4. Compared with the normal group, the level of ATF4 mRNA in the siRNA-ATF4 group was decreased significantly. Compared with the Hypoxia group, the apoptosis rate, HIF-1α and lactate dehydrogenase (LDH) contents in cells, the level of ERS was decreased in the siRNA-ATF4 treatment group. Moreover, on the basis of siRNA-ATF4 intervention group, it was found that the apoptosis rate, HIF-1α and LDH contents in cells, the level of ERS was decreased. In the *in vivo* experiment, it was first detected whether the ALF mouse model was successfully established. HE staining showed that the liver tissue structure of mice in the normal group was regular, and the liver cells were neatly arranged in the hepatic lobules. No necrosis of liver cells and inflammatory cell infiltration. The liver lobule structure of the ALF model group was destroyed by LPS combined with D-Gal, accompanied by a large number of necrotic liver cells and infiltrating inflammatory cells. Compared with normal group, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBIL) levels in serum of model group were increased. The above studies indicate that the mouse model of ALF has been successfully constructed in this study, which is consistent with previous reports. After Ars intervention, the degree of infiltration of necrotic liver cells and inflammatory cells in mouse liver tissue was reduced, and the levels of ALT, AST and TBIL in serum were decreased.

Research conclusions

Hepatocytes were damaged by hypoxia and ischemia injury in the process of ALF. At this time, the content of HIF-1α in cells increased, which inhibited the formation of SGs mediated by G3BP1 and promoted the expression of ERS marker molecules ATF4 and CHOP. The activated ERS pathway further promotes hepatocyte apoptosis. Promoting SGs synthesis can inhibit the level of hepatocyte apoptosis by inhibiting the ATF4-mediated ERS pathway.

Research perspectives

SGs could protect hepatocytes from hypoxia-induced damage during ALF by reducing ERS-mediated apoptosis.

FOOTNOTES

Author contributions: Wang Y takes responsibility for the integrity of the work as a whole, from inception to published article; Wang Y and Li WY conceived and designed the study; Li WY, Yang F, Li X and Wang LW perform the experiment; Li WY and Wang Y wrote the paper; Wang Y edited the article; all authors approved the final version of the manuscript.

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Country/Territory of origin: China

ORCID number: Xun Li 0000-0002-9598-8984; Lu-Wen Wang 0000-0002-0654-1196; Yao Wang 0000-0001-7701-5313.

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ORIGINAL ARTICLE

Observational Study

Intestinal complications in Brazilian patients with ulcerative colitis treated with conventional therapy between 2011 and 2020

Adalberta Lima Martins, Rodrigo Galhardi Gasparini, Ligia Yukie Sassaki, Rogerio Saad-Hossne, Alessandra Mileni Versut Ritter, Tania Biatti Barreto, Taciana Marcolino, Claudia Yang Santos

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Adalberta Lima Martins, Espirito Santo Health Office, State Office for Pharmaceutical Assistance, Espirito Santos 29056-030, Brazil

Rodrigo Galhardi Gasparini, Department of Gastroenterology, Specialized Medical Center, Marília 17502-020, Brazil

Ligia Yukie Sassaki, Rogerio Saad-Hossne, Department of Gastroenterology, Sao Paulo State University, Medical School, Botucatu 18618-687, Brazil

Alessandra Mileni Versut Ritter, Real World Evidence, IQVIA Brazil, Sao Paulo 04719-002, Brazil

Tania Biatti Barreto, Taciana Marcolino, Claudia Yang Santos, Department of Gastroenterology, Takeda Pharmaceuticals Brazil, Sao Paulo 04794-000, Brazil

Corresponding author: Claudia Yang Santos, BPharm, MSc, PhD, Research Scientist, Department of Gastroenterology, Takeda Pharmaceuticals Brazil, Av. das Nações Unidas, 14401 Torre Jequitiba, 11 Andar, Sao Paulo 04794-000, Brazil. clausantos2910@gmail.com

Abstract

BACKGROUND

This was an observational, descriptive, and retrospective study from 2011 to 2020 from the Department of Informatics of the Brazilian Healthcare System database.

AIM

To describe the intestinal complications (IC) of patients with ulcerative colitis (UC) who started conventional therapies in Brazil´s public Healthcare system.

METHODS

Patients ≥ 18 years of age who had at least one claim related to UC 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10) code and at least 2 claims for conventional therapies were included. IC was defined as at least one claim of: UC-related hospitalization, procedures code for rectum or intestinal surgeries, and/or associated disease defined by ICD-10 codes (malignant neoplasia of colon, stenosis, hemorrhage, ulcer and other rectum or anus disease, megacolon, functional diarrhea volvulus, intussusception and erythema nodosum). Descriptive statistics, annual incidence, and

incidence rate (IR) [per 100 patient-years (PY)] over the available follow-up period were calculated.

RESULTS

In total, 41229 UC patients were included (median age, 48 years; 65% women) and the median (interquartile range) follow-up period was 3.3 (1.8-5.3) years. Conventional therapy used during follow-up period included: mesalazine (87%), sulfasalazine (15%), azathioprine (16%) or methotrexate (1%) with a median duration of 1.9 (0.8-4.0) years. Overall IR of IC was 3.2 cases per 100 PY. Among the IC claims, 54% were related to associated diseases, 20% to procedures and 26% to hospitalizations. The overall annual incidence of IC was 2.9%, 2.6% and 2.5% in the first, second and third year after the first claim for therapy (index date), respectively. Over the first 3 years, the annual IR of UC-related hospitalizations ranged from 0.8% to 1.1%; associated diseases from 0.9% to 1.2% - in which anus or rectum disease, and malignant neoplasia of colon were the most frequently reported; and procedure events from 0.6% to 0.7%, being intestinal resection and polyp removal the most frequent ones.

CONCLUSION

Study shows that UC patients under conventional therapy seem to present progression of disease developing some IC, which may have a negative impact on patients and the burden on the health system.

Key Words: Ulcerative colitis; Brazil; Conventional therapy; Intestinal complications; Real world; Public healthcare

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Core Tip: This population-based study investigated intestinal complications (ICs) in patients with ulcerative colitis (UC) undergoing therapy available in the public healthcare system (Sistema Único de Saúde) of Brazil over the last decade. Our results showed that some patients with UC undergoing conventional therapy, seem to present an active and progressive disease and develop relevant ICs, which demand important resources of the healthcare system and have a negative impact on their lives.

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INTRODUCTION

Ulcerative Colitis (UC) is an idiopathic inflammatory disease of the mucosa and colon that commonly involve colonic regions both proximal to and distal from the site of the primary lesion[1]. The typical presentation includes symptoms such as bloody diarrhea, rectal urgency, and abdominal pain[2]. UC is a component of inflammatory bowel diseases (IBD), which are chronic, immune-mediated inflammatory diseases of the gastrointestinal tract. The etiopathogenesis is multifactorial involving different environmental, genetic, immune-mediated and intestinal microbial factors[3].

Although epidemiological studies are still scarce in developing countries, the incidence and prevalence of UC is increasing worldwide, along with IBD[4]. A systematic review showed that the incidence and prevalence of UC in Latin America varied between regions and studies, ranging from 0.04 to 8.00/100000 and 0.23 to 76.1/100000, respectively, and generally increased over the period from 1986 to 2015[5].

Many patients have long periods of complete remission, but the cumulative probability of remaining relapse-free for two years is only 20%, decreasing to less than 5% in 10 years[6]. Chronic inflammation of the colorectal mucosa, as is typically found in UC, is a proven risk factor for neoplasm of colon. The development of neoplasm of colon in patients with UC is related to several factors: Duration, extent, activity, severity and family history of the disease. The progression from UC inflammation to neoplasm and/or surgery is a multi-step process in which the accumulation of symptoms and sequential mucosal changes gradually transition to a low-grade change to neoplasm[7]. Indications for emergency surgery include refractory toxic megacolon, perforation, and ongoing severe colorectal bleeding[8].

UC is a progressive disease that can lead to other serious complications and/or even life-threatening complications. One of the most common UC disease progressions are: anorectal dysfunction (50%)[9], proximal extension (25%) with the greatest proximal extension occurring during the first 10 years[10, 11], pseudopolyposis (16%)[12] and other such as impaired permeability in remission or with mucosal healing, stenosis and dysmotility[10]. Thus, complications related to UC represent a substantial burden in terms of costs and patient quality of life, with hospital admissions, operations and treatments being the most relevant burdens[13].

The treatment of UC currently aims to monitor indicators of disease activity and to adjust therapy, aiming to achieve clinical remission and prevent long-term complications, such as dysplasia, colorectal cancer, hospitalizations and colectomy[14]. In 2021, the STRIDE-II study confirmed STRIDE-I's long-term goals of clinical remission, histological remission, and endoscopic mucosal healing[15] and added absence of disability, restoration of quality of life, and normal growth in children. Symptomatic relief and normalization of serum and fecal markers were determined as short-term targets[16].

Treatment will vary depending on the severity and location of the UC. For proctitis, topical therapy with 5-aminosalicylic acid (5-ASA) compounds is used. For more severe UC, oral and local 5-ASA compounds and corticosteroids are indicated to induce remission. For patients who do not respond to these treatments, intravenous steroids are used. When refractory to this, calcineurin inhibitors (cyclosporine, tacrolimus), tumor necrosis factor-α antibodies or immunomodulators are then used[17].

For patients with moderate to severe UC, drugs such as thiopurines and biologics are indicated to control symptoms and heal the intestinal mucosa. Anti-tumor necrotizing factor (TNF) drugs include infliximab, adalimumab and golimumab. Of the anti-integrin drug, vedolizumab can be mentioned. And the anti-p40 antibody targeting interleukin (IL)-12 and IL-23, we can mention ustekinumab and small molecules (Janus Kinase inhibitor - tofacitinib)[18]. Biologics were not available in the public healthcare system during the study period in Brazil. Studies have observed an association between the administration of biologics therapies such as anti-TNF-α within the first 2 years of diagnosis and several beneficial effects, including reduction risk of intestinal surgery[4,5,19].

There are few population-based studies documenting the evaluation and progression of the conventional therapy-using patient of the UC disease phenotype. Thus, this study aims to describe the intestinal complications (IC) of patients with UC who started treatment with conventional therapies between January 2011 and January 2020 in the Brazilian healthcare system.

MATERIALS AND METHODS

Data source

For this study, data were collected from two Department of Informatics of the Brazilian Healthcare System (DATASUS) databases: Hospitalizations Information System (SIH), which contains hospitalization data[20] and the Outpatient Information System (SIA), which contains data on outpatient care [21,22].

The causes of hospital admissions are coded according to the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10). The SIH was used to identify the number and causes of hospital admissions related to UC and/or its IC.

The SIA is the system that allows local managers to process outpatient care information recorded in outpatient care capture applications by public and private providers contracted/agreed by the public healthcare system (SUS). Most SIA subsets have an ID code that enables linking the outpatient procedures to a single patient. Because SIH does not have patient ID information, a probabilistic record linkage (RLK) was performed allowing longitudinal assessment in SIH and SIA at a patient-level. The RLK relied on multiple steps with different combination of patient information from both databases, such as date of birth, gender and ZIP code, making it possible to identify patients in both systems.

Data is presented as procedure codes from billing records and includes demographic information, all procedures such as inpatient and outpatient performance, number of procedures, and other additional information.

Study design

The present study is a descriptive, observational, retrospective study of secondary data collected between 2011 and 2020, which used the DATASUS to characterize IC in patients with UC.

We considered UC patients those who had at least one report of UC ICD-10 code (K51). The date of the first request for UC conventional therapy was considered as the index date. Conventional therapies used for UC treatment were based on procedures codes available at DATASUS, according to the following list: Sulfasalazine; Mesalazine; Azathioprine; Methotrexate; Cyclosporine. UC patients must had at least two claims of conventional therapy to be included in the study. They were followed from the index date until the last information available in the DATASUS regardless of being under conventional treatment or not. One or more conventional therapies could had been used at the same time and/or in sequence by the patient, due to that line of treatment and comparisons between different conventional therapies were not assessed in this study.

We also assessed sociodemographic, such as age, sex and location of residency of the patient. Measurements of IC information in UC patients were extracted, including procedures performed, hospitalization causes, and diseases developing in the study sample. Each pre-defined claim of IC reported after conventional therapy initiation was considered as a progression of the UC disease.

Pre-defined claims of ICs were validated based on four independent medical expert opinions and literature about the natural history and evaluation of UC[23]. Patients who progressed from UC to malignant neoplasm of colon, stenosis, hemorrhage, ulcer or other specified diseases or symptoms of anus and rectum, functional diarrhea, megacolon, volvulus, intussusception or erythema nodosum or any intestinal surgery or hospitalization related to the disease was considered to have experienced an IC.

The following claims were considered as proxy of ICs: any claim for anus, rectum or intestinal surgeries for procedures, any claim of hospitalization reporting UC ICD-10 codes for hospitalization and/or any claim of ICD-10 code pre-defined for associated diseases [Table 1, list of diseases (ICD-10 code) considered as IC in consequence of underlying UC disease].

Overall ICs could reflect one or more type of ICs, so, in an attempt to represent them, IC analyses sets were stratified into 3 groups based on the type of IC: (1) Procedures: If claim of procedure code for anus, rectum or intestinal surgeries; (2) Hospitalizations: If claim of hospitalization for UC (reported UC ICD-10 in inpatient setting); and (3) Associated disease: If claim of ICD-10 code for diseases pre-defined as UC complications, such as C18 (malignant neoplasm of colon), K62.4 (stenosis of anus and rectum), K62.5 (hemorrhage of anus and rectum), K62.6 (ulcer of anus and rectum), K62.8 (other specified diseases of anus and rectum), K62.9 (disease of anus and rectum, unspecified), K59.1 (functional diarrhea), K59.3 (megacolon, not elsewhere classified), K56.2 (volvulus), K56.1 (intussusception), or L52 (erythema nodosum). Thus, to be considered a case of IC, patients must have at least one medical complaint in one or more of the sub-groups of ICs previously described.

In the data analysis, the annual IC in UC patients per each calendar year, and the incidence per 100 people of ICs were evaluated throughout the study. Patients whose UC progressed after the conventional treatment initiation timeframe were recorded as an IC annual rate and expressed year (from 1 to 5 years) according to the number of claims (total and mean) and the number of patients (total and percentage according to the number of patients in follow-up at the database at the respective year Also, the mean ICs per each patient were assessed and expressed by calendar year. The incidence of IC was converted into units per patient/year by dividing them for all patients by the person-years of follow-up, because each individual attends a different time in the database. Thus, the incidence rate was calculated as the number of intestinal events divided by the total person-time at risk. Considering that one patient could present one or more types of IC, we also expressed the total of ICs found in the study.

The most frequent ICs related to procedures in UC population were assessed according to the number of patients with at least one procedure for anus, rectum or intestinal surgeries during the study period. The most frequent ICs related to associated disease in UC population was assessed according to the number of patients with at least one ICD-10 code of pre-defined associated-disease.

Study population

Patients were included in the study if they were aged ≥ 18 years old, if they had evidence of UC [at least one ICD-10 claim of UC (K51.0, K51.2, K51.3, K51.4, K51.5, K51.8 or K51.9)] and if they had at least one claim of conventional treatment of UC. The UC conventional therapies (synthetic) considered in the present study were sulfasalazine, mesalazine, azathioprine, methotrexate and cyclosporine.

To capture the initial stage of treatment, patients must have had no claims for conventional and/or anti-TNF therapy prior to the index date. Thus, the period from January 2010 to December 2011 was considered only for the evaluation of the medical history of patients who had the index date in 2011.

Considering that fissure and fistula of anal and rectal regions (K60) and/or abscess of anal and rectal regions (K61) are considered an uncommon IC in UC patients, but common in other IBD, we excluded patients with at least one ICD-10 claim of these diseases after index date (first claim of UC conventional treatment code). Additionally, patients with less than 6 months of follow-up in the database and patients who were not exclusive to the SUS were excluded. Non-SUS exclusive was defined as patients who used the SUS only to obtain high-cost medications and who carried out the rest of the treatment and medical care through their private health plan[24]. Thus, they are patients who had claims related to medications only.

Statistical analysis

Although only descriptive analyses were performed, a statistical review of the study was performed by a statistician. Results were described as measures of central tendency (means, medians) and spread (variance, range) for continuous variables (*e.g.*, age); absolute number and percentage for categorical variables (*e.g.*, sex) and Kaplan Meier Curve for time to event data. Ninety-five percent confidence intervals (95%CI) were calculated according to the variable, normal distribution for continuous variable, normal approximation to binominal distribution for proportion and Poisson distribution for incidence rate, as applicable.

Table 1 Description of most common intestinal complications related to associated diseases (International Statistical Classification of Diseases and Related Health Problems) or procedures (codes), *n* (%)

ICs	Description of ICs	Patients (*n* = 1273)	%	Procedure-related complications		Patients (*n* = 1125)	%
Associated disease (ICD-10–related complications)				0407020403	Abdominal rectosig-moidectomy	167	15
K62	Other diseases of the anus and rectum	1070	78	0407020390	Body removed – rectum or colon polyps	145	13
C18	Malignant neoplasms of the colon	255	19	0407020063	Partial colectomy	135	12
K59	Other functional intestinal disorders	66	5	0407020209	Enteroctomy and/or resection	128	11
L52	Erythema nodosum	27	2	0407020101	Colostomy	105	9
K56	Paralytic ileus and intestinal obstruction without hernia	3	0.2	0407020128	Dilatation of the anus and/or rectum	91	8
K51	Ulcerative colitis	2	0.1	0407020098	Abdominal colporrhaphy	51	5
C63	Malignant neoplasms of other and unspecified male genital organs	1	0.1	0407020241	Enterostomy closure	50	4
				0407020187	Enteroanastomy	41	4

Enterotomy is surgical incision into the intestine. ICs: intestinal complications; ICD: International Classification of Diseases.

The age variable was calculated based on the difference between the date of birth and the first conventional therapy claim reported (index date). The age was described as continuous variable, including the mean, standard deviation, median and quartiles. For the sex and race variables, they were described as categorical variables, with absolute frequencies and percentage.

Time of follow-up was calculated based on the difference between date of first claim of conventional therapy (index date) and the last date of patient information available at database. Conventional treatment profile of UC patients was evaluated and expressed by the percentage of patient who had at least one claim of UC therapy. Patients could change, add or stopped some of the conventional therapies (sulfasalazine, mesalazine, azathioprine, methotrexate and cyclosporine) during the treatment approach, therefore, the patient may have used one or more conventional therapies during the study period. Conventional treatment initiation was expressed as the number of patients (absolute and percentage) per calendar year (2011-2020) according to the first claim of the conventional therapy in the database. Timing using conventional therapy was calculated based on the first and the last claim of the therapy presented by the patient regardless of gaps. Switch conventional therapy was considered when it had at least one claim of different conventional therapy than the previous one. The time between one conventional therapy to another conventional therapy was described as continuous variable, including the mean, standard deviation, median and quartiles of the mean.

The main outcomes were regarding ICs in patients who initiated on conventional treatment between 2011 and 2020 in the SUS. Since each individual attended different amount of time in the database, some ICs parameters were analyzed as the frequency per person-years of follow-up. For incidence rate, confidence interval was estimated by Poisson rate confidence interval. The number of claims and the number of patients with at least one claim were expressed to address the total number of claim [patient could present more than one IC (claim) for the respective group of IC] and the total number of patients with at least one IC (claim).

Missing values were reported as missing information and imputation methods were not used. When needed, patient was censored for IC analyses. For Kaplan-Meier curve the last information available of the patient or the end of the study period was considered as censored for patients who did not present IC. IC was not assessed according to the conventional treatment received and neither if patient discontinued or not discontinued the conventional therapy treatment. Data was analyzed using Python version 3.6.9.

RESULTS

In the present study, a total of 41229 patients with UC meeting the inclusion criteria were exposed to conventional medications between 2011 to 2020 in DATASUS. Most patients were female (65%) and

were reported as white (56%). The mean ± SD age was 48 ± 15.42 years old, with a mean ± SD follow-up of 3.65 ± 2.27 years. Most patients (56%) were in the Southeast region of Brazil, followed by South (21%), and Northeast (14%) (Table 2).

All patients with UC had received conventional therapy and the mean ± SD time using each was 2.64 ± 2.29 years. Regarding the conventional treatment profile for patients with UC, mesalazine had the highest proportion (87%). Azathioprine was the second (16%) and sulfasalazine the third (15%) most frequent drugs among patients with UC. Methotrexate was reported only by 1% of the patients and cyclosporine by less than 1% (Table 2).

Table 3 shows the incidence rate of bowel complications in patients with UC exposed to conventional treatment. The overall IR was 2.55 (2.46-2.63) per 100 patients. The IR of associated diseases (ICD-10-related), procedures-related and UC hospitalizations were 0.93 (0.89-0.98), 0.76 (0.71-0.80) and 0.97 (0.92-1.02), respectively. Considering that one patient could present more than one IC, a total of 6711 bowel complications claims were reported over the study period in these patients (Table 3).

Regarding the annual IC (overall and segregated types) in patients with UC, during the study period (≤ 1 year and 5 years), the overall complications rates were 2.90% at one year and 2.36% at 5 years. Segregated by type, complications related to the associated diseases (ICD-10) were1.18% and 0.88%; the procedure-related complications were 0.71% and 0.68%; and the complications related to hospitalizations due to colitis was 1.10% and 0.84%, respectively (Table 4). The mean number of events per patient was also similar over the years for both the overall and the segregated IC types in patients with UC (Table 4). A description of the time to event for overall and segregated IC type in the population with UC is shown in Figure 1. In the Kaplan-Meier analysis, the probability to present any type of ICs (overall) was approximately 20% by the end of the study period. The segregated IC types had a comparable probability to present an event (less than 10% for procedures, hospitalization and associated disease) by the end of the study period (Figure 2).

From the 1273 patients with IC due to disease associated to UC found in the study, the categories Other diseases of the anus and rectum (78%), Colon malignant neoplasm (19%), Other functional intestinal disorders (5%) were the most common ones (Table 1).

Additionally, for the 1125 patients with IC due to procedures performed in consequence of the UC progression, it was found that abdominal rectosigmoidectomy (15%), body removed-rectal or colon polyps (13%) and partial colectomy (12%) were the most common ones (Table 1).

The length of hospitalization stay was 6.62 ± 6.62 d and the number of procedures performed during a hospitalization was 0.32 ± 0.28. The most common hospitalization procedure was the treatment of non-infectious enteritis and colitis (77%), followed by diagnosis and/or emergency care at a medical clinic (7%) and treatment of other intestinal diseases (4%) (Table 5).

DISCUSSION

The present study described some specific categories of ICs in patients with UC treated with conventional therapies available in the public health system in Brazil between January 2011 and January 2020. Women, who were reported as white and from the Southeast region were the patients with the highest proportions in this study. The mean time of use of conventional therapy was 2.64 years and mesalazine was the most conventional therapy used. The mean age of UC patients treated with conventional medication in SUS was 48.86 years. This finding is similar to another database study, which reported that the highest proportion of patients with UC was in the 45-54 age group[25].

UC is considered a global burden due to its high prevalence and incidence in developed countries. Lima Martins *et al*[26], in 2014, carried out a study in Brazil, presenting a UC incidence of 5.3/100000 inhabitants/year. In São Paulo, Brazil the incidence of UC was 8/100000 inhabitants per year in 2015 [27]. A study carried out in an underdeveloped region of Northeastern in Brazil found an incidence of UC of approximately 0.6/100000 inhabitants per year in 2012[28].

Demographic characteristics of patients with UC in DATASUS showed that the large proportion of patients were from the Southeast region and the minority from the North region of Brazil. Although these regions represent one of the highest and lowest densities in the country, respectively, the number of UC cases in each region may also be due to social differences and inequalities in access to health services found in the country, in line with previous Brazilian perspectives findings reported in the literature[29].

Several studies have shown that uncontrolled UC is associated with structural damage and impaired gastrointestinal functioning[30]. This population-based study in Brazil depicted the year-by-year proportion of patients with IC under conventional therapy in SUS. During the first year and the last 5 years of follow-up, about 2.90% and 2.36%, respectively, of the patients had one or more IC in consequence of the UC progression. The rate of IC also appeared not to be high, similarly found in another international study. Thus, the proportion of patients with some complication seems to be constant over time even under treatment. It is known that continuing conventional therapy in some cases may delay more effective therapy and place patients at risk for worsening disease and complications[31].

Table 2 Demographic characteristics and follow-up of patients with ulcerative colitis treated with conventional therapies, *n* (%)

Variable	*n*	%
Patients	41229	100
Age[1], yr		
mean ± SD	48.86 ± 15.42	
Median (IQI)	48.09 (60.15-36.94)	
Sex		
Male	14417	35
Female	26812	65
Ethnicity		
White	23290	56
Black and other	11987	33
Missing	4643	11
CVT initiation		
2011	4300	10
2012	4054	10
2013	4259	10
2014	4877	12
2015	4846	12
2016	5039	12
2017	5565	13
2018	5040	12
2019	3249	8
Follow-up time[2], yr		
mean ± SD	3.65 (2.27)	
Median (IQI)	3.25 (1.75-5.26)	
Region of residence		
Southeast	22942	56
South	8831	21
Midwest	2638	6
Northeast	5833	14
North	985	2
Missing	0	0
Patients under CVT		
Mesalazine	35923	87
Sulfasalazine	6148	15
Azathioprine	6413	16
Ciclosporine	116	0
Methotrexate	290	1
Time using CVT[3], yr		
mean ± SD	2.64 (2.29)	
Median (IQI)	1.92 (0.75-4.00)	
Patients switched treatment after first	2564	6

IC[4]	
Time to switch treatment after IC, yr	
mean ± SD	0.36 (0.76)
Median (IQI)	0.08 (0.08-0.25)
Min	0.08

Values are expressed as numbers (%) or medians (range).
[1]Age at index date.
[2]Time since index date up to the last date of patient information available in the database.
[3]Calculated based on the index date and the last claim of conventional therapy.
[4]Switching from one conventional therapy to another conventional therapy.
CVT: Conventional therapy; IQI: Interquartile interval; UC: Ulcerative colitis.

Table 3 Incidence of intestinal complications (overall and segregated types) in patients with ulcerative colitis considering all population at risk

	Total of events	First event[1] (*n*)	PY[2]	IR (95%CI)
Overall	6711	3616	141997.40	2.546 (2.464-2.628)
Associated disease (ICD-10-related)	3766	1372	146806.44	0.934 (0.885-0.983)
Procedure-related	1324	1125	148326.75	0.758 (0.714-0.802)
Colitis-related Hospitalization	1791	1426	147412.62	0.967 (0.917-1.017)

Incidence rate = *n* of patients with event/patient year × 100.
[1]Counted only the first intestinal complication (claim), but the patient could present with more than one IC (represented by the sum of the first claim).
[2]Patient year = sum of time of all patients under risk, since index date up to first IC or last available data.
ICs: Intestinal complications; IR: Incidence rate; ICD: International Classification of Diseases; PY: Patient year; UC: Ulcerative colitis.

Although the rates of ICs in the present study might be underestimated due to intrinsic limitation of the method applied, the impact of the progression of UC disease with consequent complication for the health system and patient might be significant, especially due to the severity and type of the complication generated for the patient. We found that almost 4% of the UC patients performed a surgery and/or procedure of anus or rectum during the study period. Surgical complications represent a substantial burden in terms of cost and quality of life, with reoperations, medical fees and additional hospital admissions being the main cost factors[13].

The associated diseases observed in the present study were mainly represented by other diseases of rectum and anus (78%) followed by the malignant neoplasm of colon (19%). Literature shows that UC patients with severe and persistent inflammation have an increased risk of developing neoplasm of colon[32]. In the United States, the prevalence of neoplasm of colon in a patient with UC was about 3.7% [33], in Spain was 11.7%[34]. It is well known that cancer is more likely to develop in patients with extensive colitis lasting 8-10 years or more[35]. In our investigation, the average follow-up period was only 3.65 years. Thus, it is possible that our results would be improved by a longer follow-up period.

Although no direct statistical comparison was performed, the incidence rates of ICs found in the present study indicate that ICs related to diseases associated to UC (ICD-10 code) were the highest, followed by those related to the procedure and finally hospitalization for UC. Only hospitalization related to ICD-10 of UC was considered in this study, which may imply underestimated rates. That is, if the patient was hospitalized due to the progression of the disease, other ICDs-10 codes were probably used and, therefore, was not counted in this study. Even though, we might assume that conventional therapy was not able to control the inflammatory process for all patients leading them to seek urgent and emergency services, or even been hospitalized and/or require surgeries procedures.

Conventional medical treatment for UC may have limited efficacy or serious adverse reactions[36]. Currently, 20% to 40% of patients with UC do not respond to conventional therapies and may receive secondary drug treatment or even colectomy. Anti-TNF agents were the first biologics to be used in the treatment of IBD, including infliximab and adalimumab. Other biologics therapies that target specific immune pathways has been implemented in several countries, including in Brazil, as potential therapeutics for UC[37,38]. In this line, Vedolizumab was recently approved by ANVISA for the treatment of adult patients with moderate to severe UC[39] who are not responding to one or more conventional treatments, such as steroids, immunosuppressive agents or TNF blockers[36]. However, during the study period, there was no biological therapy available in the SUS.

Table 4 Annual incidence of intestinal complications (overall and segregated type) in patients with ulcerative colitis over the study period (January 2011-February 2020)

	Overall				Associated disease (ICD-10–related)				Procedure-related				UC-related hospitalization			
	Patients (*n*)	%	Claims (*n*)	mean ± SD	Patients (*n*)	%	Claims (*n*)	mean ± SD	Patients (*n*)	%	Claims (*n*)	mean ± SD	Patients (*n*)	%	Claims (*n*)	mean ± SD
≤ 1 yr (*n* = 41229)	1197	2.90	1582	1.32 ± 1.3	487	1.18	763	1.57 ± 1.92	293	0.71	312	1.06 ± 0.29	456	1.10	520	1.14 ± 0.44
2 yr (*n* = 36389)	931	2.56	1250	1.34 ± 1.35	355	0.98	601	1.69 ± 2.08	262	0.72	284	1.08 ± 0.36	343	0.94	376	1.1 ± 0.32
3 yr (*n* = 28795)	716	2.49	953	1.33 ± 1.39	305	1.06	493	1.62 ± 2.05	182	0.63	201	1.1 ± 0.34	242	0.84	263	1.09 ± 0.34
4 yr (*n* = 22012)	531	2.41	668	1.26 ± 1.16	219	0.99	323	1.47 ± 1.72	158	0.72	170	1.08 ± 0.37	163	0.74	177	1.09 ± 0.32
5 yr (*n* = 16510)	389	2.36	457	1.17 ± 0.88	146	0.88	190	1.3 ± 1.36	113	0.68	115	1.02 ± 0.13	139	0.84	155	1.12 ± 0.36

n of patients: Counted only the number of patients with intestinal complications (ICs) (first IC), but patients could present with more than one IC. *n* of claims: Counted the total number of claims. ICs: Intestinal complications; ICD: International Classification of Diseases; IR: Incidence rate; UC: Ulcerative colitis.

The treatments available in the SUS until 2020 involved the use of aminosalicylates (sulfasalazine and mesalazine), corticosteroids (hydrocortisone and prednisone), immunosuppressants (azathioprine, 6-mercaptopurine and (intravenous cyclosporine), and antibiotics[40]. Biological therapy was included in the national protocol of UC treatment in early 2020[40,41]. Before that period, patients who needed biological therapy had to find other strategies besides the SUS to get biologic therapies for UC treatment, such as out of pocket, private healthcare and/or even judicial action.

Limitations

The main limitations of this study are related to the retrospective design of the study and the potential information biases and attrition of this study. With regard to information bias, the lack of information can lead to underestimation of results. Longitudinal and time-dependent analyzes of data correction are more accurate, however, DATASUS data end up being limited by this. Another limitation refers to the availability of medicines by the SUS, which are not always available and/or are available at different times during the period of this study, such as corticosteroids. Use of conventional therapy was an assumption based on the first claim of each drug at the database, so the patients could have not been under treatment during all the study period. However, as a real word study, this scenario reflects the daily life of UC patients. The severity of the disease was not considered in the analyses due to methodological limitations.

Additionally, for data analysis, the inpatient database (SIH) does not have patient identification and the use of probabilistic RLK, event rates may be underestimated, as they are mainly captured in outpatient databases (SIA). As it is a descriptive observational study, it is prone to confounding factors that are difficult to control or assess. In an attempt to reduce these biases, broad terms of procedures and ICD-10s related to possible complications were used. Although the ICD-10 codes used, procedures and hospitalization were predefined as ICs, other diseases and/or procedures related to UC could be codes could have been studied as ICs proxy, however, we were not able to predict all ICD-10.

Table 5 Length of hospitalization and most common procedures performed during hospitalization

Length of hospitalization and procedures	mean ± SD	Median (IQI)	Procedures (inpatient most common procedures[1])		Patients (*n* = 1426)	%
			ICs	Description of ICs		
Hospitalization (inpatient)			303070099	Treatment of enteritis and colitis not infectious	1103	77
Length of hospital stay, d (PPPY)	6.62 ± 6.62	4.60 (2.63-8.11)	301060088	Diagnose and/or emergence care in medical clinic	102	7
Number of procedures performed (PPPY)	0.32 ± 0.28	0.23 (0.15-0.38)	303070110	Treatment of other intestinal diseases	56	4
			301060070	Diagnosis and/or emergence care in surgery clinic	47	3
			415010012	Treatment with multiple surgeries	44	3
			415020034	Other procedures with following surgeries	30	2
			407020071	Total colectomy	29	2
			303070102	Treatment of digestive tract disease	23	2
			407020330	Total proctocolectomy	12	1
			303010061	Exploratory laparotomy	9	1

[1]Inpatient procedures reported with ulcerative colitis disease International Statistical Classification of Diseases and Related Health Problems codes.
ICs: Intestinal complications; ICD: International Classification of Diseases; IQI: Interquartile interval; PPPY: Per patient per year; UC: Ulcerative colitis.

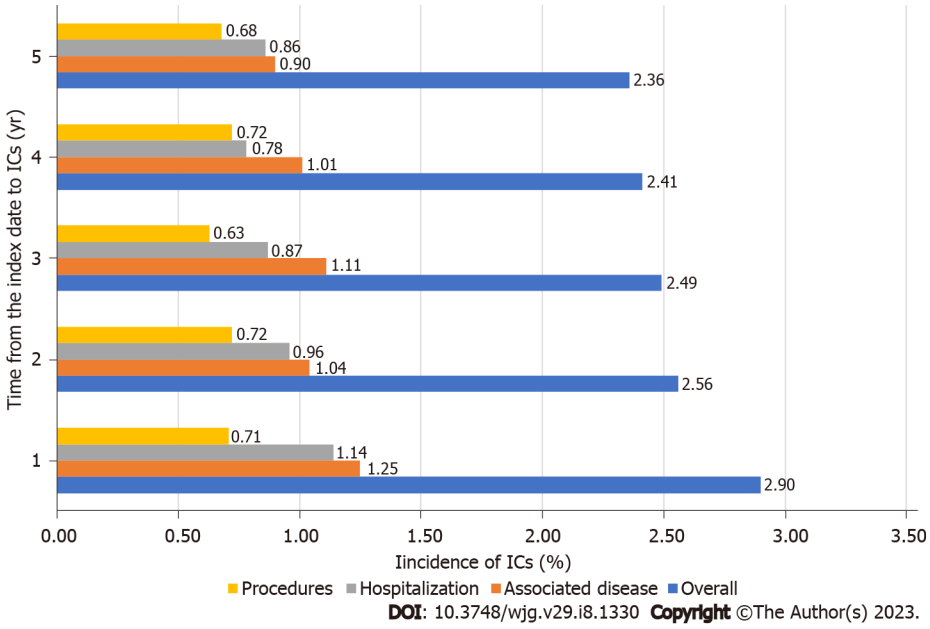
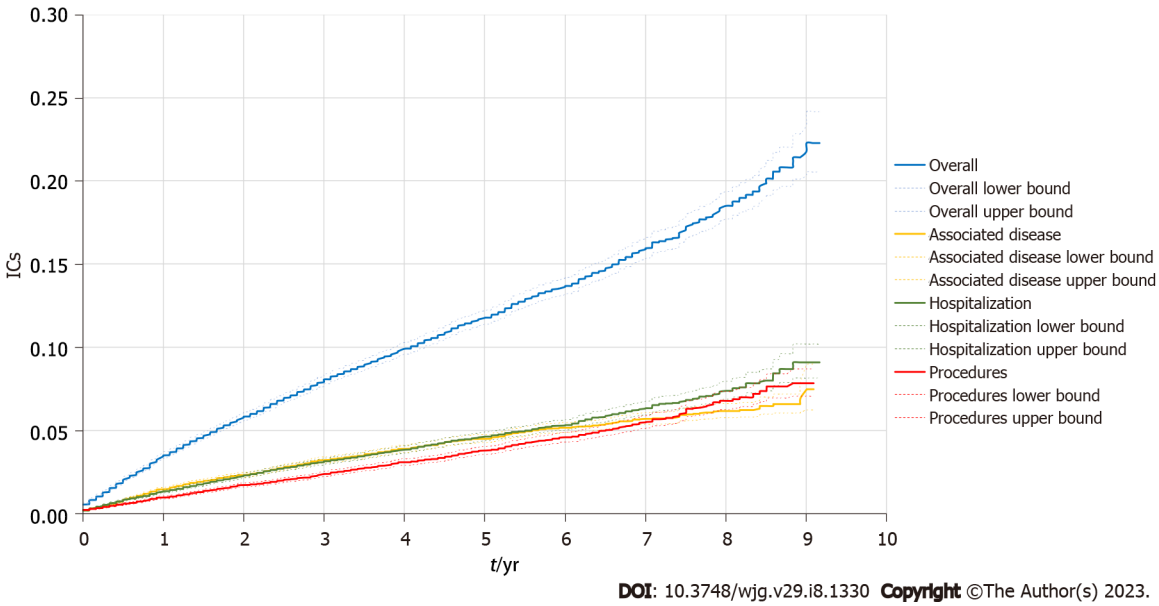


Figure 1 Annual intestinal complication (overall and segregated types) in ulcerative colitis patients over the study period (January 2011-February 2020). ICs: Intestinal complications.

CONCLUSION

UC have impact both for the population and public system (SUS) due to its clinical management and development of comorbidities and associated diseases. The results of this study highlight some issues regarding the progression of UC in patients who were treated with conventional therapies in the Brazilian public system showing that a percentage of UC patients have presented IC during the history of the disease. Most of the IC represent an important demand to the health system, with a need for health support and hospitalization. Current UC therapies, as well as innovative therapies that effectively alleviate UC symptoms, along with the implementation of an adequate program to access



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Figure 2 Kaplan-Meier curve depicting time to intestinal complication in ulcerative colitis patients. ICs: Intestinal complications.

and manage therapy for patients with UC, may improve the control or progression of UC and consequently decrease the negative the impact of the disease.

ARTICLE HIGHLIGHTS

Research background

This was an observational, descriptive, and retrospective study from 2011 to 2020 from the Department of Informatics of the Brazilian Healthcare System database.

Research motivation

To understand the real world situation of patients with ulcerative colitis (UC) in the public healthcare system.

Research objectives

Describe the intestinal complications (IC) of patients with UC who started conventional therapies.

Research methods

Patients ≥ 18 years of age who had at least one claim related to UC International Statistical Classification of Diseases and Related Health Problems (ICD-10) code and at least 2 claims for conventional therapies were included. IC was defined as at least one claim of: UC-related hospitalization, procedures code for rectum or intestinal surgeries, and/or associated disease defined by ICD-10 codes.

Research results

In total, 41229 UC patients were included. Overall IR of IC was 3.2 cases per 100 PY. Among the IC claims, 54% were related to associated diseases, 20% to procedures and 26% to hospitalizations. The overall annual incidence of IC was 2.9%, 2.6% and 2.5% in the first, second and third year after the first claim for therapy (index date), respectively. Over the first 3 years, the annual IR of UC-related hospitalizations ranged from 0.8% to 1.1%; associated diseases from 0.9% to 1.2%.

Research conclusions

Study shows that UC patients under conventional therapy seem to present progression of disease developing some IC, which may have a negative impact on patients and the burden on the health system.

Research perspectives

Biologics were implemented in the public healthcare system after the study period, it would be interesting to verify the IC impact of this implementation in patients with UC.

FOOTNOTES

Author contributions: Martins AL, Galhardi Gasparini R, Sassaki LY, Saad-Hossne R, Barreto TB, Marcolino T and Yang Santos C participated in designed, interpretation of the data and revised the article critically for important intellectual content; Ritter AMV participated in the acquisition, analysis and draft the initial manuscript.

Institutional review board statement: In alignment with Brazilian ethical resolution number 510, 2016, ethical approval was not necessary since this is a secondary study using anonymized data.

Conflict-of-interest statement: Martins AL served on the advisory board of Takeda, AbbVie, Janssen, Pfizer and Amgen and is a speaker for Amgen and Janssen. Galhardi Gasparini R is a speaker for Janssen, Takeda and AbbVie. Sassaki LY is a speaker for Janssen and Takeda and participated in the advisory boards of Takeda and AbbVie. Saad-Hossne R is a speaker and on the advisory boards for AbbVie, Takeda, Janssen, Pfizer, Fresenius and Amgen, as well as a speaker for Novartis. AMVR is an employee of IQVIA Brazil. Marcolino T, Barreto TB and Yang Santos C are employees of Takeda Pharmaceutical Brazil.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE statement, and the manuscript was prepared and revised according to the STROBE statement.

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Country/Territory of origin: Brazil

ORCID number: Adalberta Lima Martins 0000-0002-0273-5743; Rodrigo Galhardi Gasparini 0000-0002-1032-5349; Ligia Yukie Sassaki 0000-0002-7319-8906; Rogerio Saad-Hossne 0000-0002-8166-0304; Alessandra Mileni Versut Ritter 0000-0002-4434-4791; Tania Biatti Barreto 0000-0003-1871-1815; Taciana Marcolino 0000-0003-2578-4537; Claudia Yang Santos 0000-0002-6023-607X.

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ORIGINAL ARTICLE

Observational Study

Establishment of a prediction model for severe acute radiation enteritis associated with cervical cancer radiotherapy

Chen-Ying Ma, Jing Zhao, Guang-Hui Gan, Xiao-Lan He, Xiao-Ting Xu, Song-Bing Qin, Li-Li Wang, Li Li, Ju-Ying Zhou

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Chen-Ying Ma, Jing Zhao, Guang-Hui Gan, Xiao-Lan He, Xiao-Ting Xu, Song-Bing Qin, Li-Li Wang, Li Li, Ju-Ying Zhou, Radiation Oncology, The First Affiliated Hospital of Soochow University, Suzhou 215123, Jiangsu Province, China

Ju-Ying Zhou, State Key Laboratory of Radiation Medicine and Protection, Soochow University, Suzhou 215123, Jiangsu Province, China

Corresponding author: Ju-Ying Zhou, MD, Radiation Oncology, The First Affiliated Hospital of Soochow University, No. 188 Shizi Street, Suzhou 215123, Jiangsu Province, China.
zhoujuyingsy@163.com

Abstract

BACKGROUND

Cervical cancer is one of the most common gynecological malignant tumors. Radiation enteritis (RE) leads to radiotherapy intolerance or termination of radiotherapy, which negatively impacts the therapeutic effect and seriously affects the quality of life of patients. If the incidence of RE in patients can be predicted in advance, and targeted clinical preventive treatment can be carried out, the side effects of radiotherapy in cervical cancer patients can be significantly reduced. Furthermore, accurate prediction of RE is essential for the selection of individualized radiation dose and the optimization of the radiotherapy plan.

AIM

To analyze the relationships between severe acute RE (SARE) of cervical cancer radiotherapy and clinical factors and dose-volume parameters retrospectively.

METHODS

We included 50 cervical cancer patients who received volumetric modulated arc therapy (VMAT) from September 2017 to June 2018 in the Department of Radiotherapy at The First Affiliated Hospital Soochow University. Clinical and dose-volume histogram factors of patients were collected. Logistic regression analysis was used to evaluate the predictive value of each factor for SARE. A nomogram to predict SARE was developed (SARE scoring system ≥ 3 points) based on the multiple regression coefficients; validity was verified by an internal verification method.

RESULTS

Gastrointestinal and hematological toxicity of cervical cancer VMAT gradually increased with radiotherapy and reached the peak at the end of radiotherapy. The main adverse reactions were diarrhea, abdominal pain, colitis, anal swelling, and blood in the stool. There was no significant difference in the incidence of gastrointestinal toxicity between the radical and postoperative adjuvant radiotherapy groups ($P > 0.05$). There were significant differences in the small intestine V_{20}, V_{30}, V_{40}, and rectal V_{40} between adjuvant radiotherapy and radical radiotherapy after surgery ($P < 0.05$). Univariate and multivariate analyses revealed anal bulge rating (OR: 14.779, 95%CI: 1.281-170.547, $P = 0.031$) and disease activity index (DAI) score (OR: 53.928, 95%CI: 3.822-760.948, $P = 0.003$) as independent predictors of SARE.

CONCLUSION

Anal bulge rating (> 0.500 grade) and DAI score (> 2.165 points) can predict SARE. The nomogram shows potential value in clinical practice.

Key Words: Cervical cancer; Intensity-modulated radiotherapy; Radiation enteritis; Nomogram; Predictor

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Core Tip: Radiation enteritis (RE) not only seriously affects the quality of life of patients, but it also leads to radiotherapy intolerance or termination of radiotherapy. The aim of our study was to determine the cumulative incidence of acute RE associated with cervical cancer radiotherapy in patients with RE in organs at risk and changes in dose-volume histogram indices. The nomogram of severe acute RE (SARE) was further developed according to the clinical factors, cumulative incidence of SARE and dosimetric parameters of volumetric modulated arc therapy patients, which may be useful for individualized risk assessment and accurate prediction of SARE to guide clinical treatment strategies.

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INTRODUCTION

Cervical cancer is one of the most common gynecological malignant tumors, ranking fourth among malignant tumors in females worldwide[1]. Radiotherapy plays an important role in the postoperative adjuvant treatment of cervical cancer patients and the radical treatment of patients who are ineligible for surgery. Radiation enteritis (RE) is a common and potentially dose-limiting toxic reaction of pelvic radiotherapy. The clinical manifestations include nausea, vomiting, abdominal pain, bloody stool, mucous stool, diarrhea, tenesmus, and urinary incontinence. RE not only seriously affects the quality of life of patients, but it also leads to radiotherapy intolerance or termination of radiotherapy, which negatively impacts the therapeutic effect. If the incidence of RE in patients can be predicted in advance, and targeted clinical preventive treatment can be carried out, the side effects of radiotherapy in cervical cancer patients can be significantly reduced. Furthermore, accurate prediction of RE is essential for the selection of individualized radiation dose and the optimization of the radiotherapy plan.

Many studies have shown that the occurrence and development of RE are related to intestinal radiotherapy technology, total dose, total volume, fraction dose, ratio of dose to volume, and uniformity of radiation dose distribution[2-6]. RE is also influenced by factors such as inflammatory bowel disease [7], collagen vascular disease[8], history of abdominal and pelvic surgery[9], history of pelvic inflammatory disease, diabetes mellitus[10], and acquired immunodeficiency syndrome[11]. However, there is no consensus on the relative importance of these predictors. Only a few studies have explored the dosimetric risk factors for RE in cervical cancer patients[12]. How to predict RE and reduce adverse reactions is the core issue of clinical radiotherapy for cervical cancer. Notably, a single predictor has limited predictive power and cases can show substantial differences in heterogeneity of RE; these issues should be addressed in further research[13]. To specifically and accurately identify RE, there is an urgent need for an accurate predictive model that combines multiple factors.

The initial objective of this study was to determine the cumulative incidence of acute RE associated with intensity modulated radiation therapy (IMRT) for cervical cancer in patients with RE in organs at risk (OAR) and changes in dose-volume histogram indices. The severe acute RE scoring system (SARE-

SS) was defined by integrating clinical factors and dosimetric physical parameters. Finally, the nomogram of severe acute RE (SARE) was further developed according to the clinical factors, cumulative incidence of SARE and dosimetric parameters of volumetric modulated arc therapy (VMAT) patients. This nomogram may be useful for individualized risk assessment and accurate prediction of SARE to guide clinical treatment strategies.

MATERIALS AND METHODS

Patient selection

Fifty patients with cervical cancer who received pelvic local radiotherapy in the Department of Radiation Oncology of The First Affiliated Hospital of Soochow University from August 2017 to August 2018 were selected. The inclusion criteria were as follows: (1) 18-80 years old; (2) cervical cancer confirmed by pathology; (3) no history of intestinal diseases or metabolic diseases; and (4) good understanding and communication skills. The exclusion criteria were as follows: (1) Severe heart, liver and kidney dysfunction; (2) termination of treatment because of serious complications during and after radiotherapy (such as cardiopulmonary, hepatic and renal insufficiency, severe infection, severe bone marrow suppression, massive hemorrhage, vaginorectal fistula and vaginovesical fistula); and (3) refusal to participate in the study.

This study was approved by the ethics committee of The First Affiliated Hospital of Soochow University Medical Ethics Committee [approval No. 2016(100)]. All enrolled patients provided signed informed consent.

External irradiation method for cervical cancer

Radiotherapy simulation positioning: On the night before radiotherapy, all patients took laxatives to clean the intestinal track. One hour before positioning, the bladder was emptied and patients drank 800 mL of water to fill the bladder. The patients then took the supine position. A Philips [Brilliance computed tomography (CT) Big Bore] large aperture CT machine was used for simulated CT localization, and enhanced CT scanning was performed with a slice thickness of 5 mm. The scanning range was from the upper edge of T11 vertebral body to 5 cm below the ischial tuberosity. The CT localization images were transferred to the treatment planning system (V5.1.1, Elekta, Monaco, Sweden).

Radiotherapy target delineation: Magnetic resonance imaging (MRI), CT or positron emission tomography-CT (PET-CT) examinations were routinely performed during the pre-radiotherapy evaluation. The target volume and OAR were delineated according to the standards of Radiation Therapy Oncology Group and Japan Clinical Oncology Group.

Radiotherapy plan design: The Monaco treatment planning system was used to design the 7-field reverse dynamic VMAT plan using unified parameters. The X-ray beam energy was 6 MV. At least 95% of the pelvic lymphatic drainage (PTV) is required to reach the prescribed dose, and no hot spot of ≥ 110% dose can occur outside the PTV. The OAR dose limit was uniform (OAR dose limitation: Bladder $V_{40} < 0\%$, rectum $V_{40} < 40\%$, colon $V_{40} < 30\%$, small intestine $V_{40} < 20\%$, femoral head $V_{45} < 5\%$).

Prescription dose of the VMAT target volume after operation: We administered 45 Gy/25 fractions for moderate risk included lymph node metastasis, paracytal invasion, and positive margin) PTV1 and 50 Gy/25 fractions for high risk PTV1 50. 4 Gy/28 fractions, the prescription dose of the remaining target volume can be executed according to the radical external irradiation scheme.

Prescription dose of the radical VMAT target volume: We administered the following doses: PTV1 45 Gy/25 fractions, parametrial area (PTV2) 50 Gy/25 fractions, and positive lymph nodes (PGTVnd) 56 Gy/28 fractions. In cases with para-aortic area, the dose for the para-aortic lymphatic drainage area (PTV3) was 36-40 Gy/20 fractions.

Implementation of the VMAT plan: VMAT was performed using the Elekta Synergy linear accelerator. All patients were treated five times a week, with one treatment a day. Cone-beam CT (Elekta) image-guided VMAT was performed at least once a week, and the error of each treatment was kept within 3 mm.

Chemotherapy for cervical cancer

Of the 50 cervical cancer patients included in this study, 18 were treated with concurrent chemoradiotherapy (CCRT) and 32 received sequential chemoradiotherapy (SCRT). All doses and adjustments of the chemotherapy regimen followed the NCCN guidelines[14].

End point definitions

General data collection: Data on age, weight, height, body mass index (BMI)[15], age-adjusted Charlson Comorbidity Index (aCCI), pathological data, HPV, squamous cell carcinoma antigen, risk rating, FIGO

2018 staging information, surgery, radiotherapy and chemotherapy were collected.

Data collection of acute RE: The adverse reactions of all patients were monitored at baseline and 2 wk, 4 wk, and 6 wk after the start of radiotherapy. Adverse reactions included diarrhea, abdominal pain, colitis, anal distension, hematochezia and bone marrow suppression. The diagnosis and classification of RE were determined following the National Institutes of Health Common Adverse Events Evaluation Criteria (CTCAE 5.0).

We established the SARE-SS to reflect the adverse reaction score as follows: SARE-SS = the sum of CTCAE scores of diarrhea + abdominal pain + colitis + anal bulging + hematochezia. A score of ≥ 3 indicated the presence of SARE. The diagnosis of SARE was determined by at least two experienced radiation oncologists on the basis of clinical symptoms and changes in the results of ancillary tests.

Disease activity index determination: The disease activity index (DAI) is based on scores that reflect the weight loss of patients (weight unchanged is 0, 1%-5% is 1 point, 5%-10% is 2 points, 10%-15% is 3 points, more than 15% is 4 points), stool viscosity (normal is 0 points, loose stool is 2 points, diarrhea is 4 points) and stool bleeding (normal is 0 points, occult blood positive is 2 points, dominant bleeding 4 points). The sum of the three scores is divided by 3 to obtain the final DAI score.

Radiotherapy planning parameter collection: Statistics of different OAR dose-volume relationship (percentage of PTV volume receiving prescription dose), recorded as V_x.

Statistical analysis

Statistical analysis was performed using SPSS 20.0. The quantitative data of normal distribution were expressed as (mean ± SD), and *t* test was used for comparisons between groups. The data of skew distribution were tested by Mann-Whitney *U* test. Qualitative data were expressed as number of cases and percentage; Fisher's exact probability method or χ^2 test was used to compare unordered categorical data, and Mantel-Haenszel χ^2 and Mann-Whitney *U* test were used to compare ordered categorical data. $P < 0.05$ was considered to indicate statistical significance. Receiver operating characteristic curve (ROC curve) was used to analyze the specificity and sensitivity of OAR dose-volume parameters in predicting RE. Two-factor repeated-measures ANOVA was used to analyze the relationship between DAI scores and time in different groups, and one-way ANOVA was used to compare the differences of DAI scores among four time points in each group.

To establish the prediction model, univariate logistic regression model was used to evaluate the predictive ability of each factor for SARE. Multivariate analysis was performed for significant factors from the univariate analysis. Kendall correlation analysis was used to avoid multicollinearity between factors. Factors with significant predictive value in the multivariate analysis were used to construct a nomogram. The area under the ROC curve (AUC), calibration curve and decision curve analysis (DCA) were used for nomogram validation. Data analysis was performed using R software (software version 4.0.2, R package version rmda 1.6).

RESULTS

Clinical characteristics of patients

This study included 50 patients with cervical cancer. Among the 50 patients, 14 patients received radical radiotherapy, 35 patients received postoperative adjuvant pelvic radiotherapy and 1 patient received pelvic radiotherapy combined with after loading vaginal radiotherapy because the vaginal margin was 2 cm away from the tumor boundary. The specific clinical factors are detailed in Table 1. The mean age of the postoperative adjuvant radiotherapy group was significantly lower than that of radical radiotherapy group ($P < 0.05$) (Table 2). The BMI of the postoperative adjuvant radiotherapy group was also significantly lower ($P < 0.05$), but there was no significant difference in weight, pathological type, cumulative incidence of RE and SARE and irradiation of para-aortic extension field between the two groups ($P > 0.05$).

Dose-volume comparison of OAR in the VMAT plan between the postoperative adjuvant radiotherapy group and radical radiotherapy group

Patients in the radical radiotherapy group were treated with total pelvic and parauterine local radiotherapy, and patients in the postoperative adjuvant radiotherapy group were treated with total pelvic prophylactic radiotherapy. The dose and volume of small intestine in the radical radiotherapy group were significantly reduced compared with that in the postoperative adjuvant radiotherapy group, and the decrease of V_{20}, V_{30} and V_{40} were statistically significant ($P < 0.05$) (Table 3). Rectal V_{30}, V_{35}, and V_{40} in the radical radiotherapy group were significantly higher than those in the postoperative adjuvant radiotherapy group. The increase of V_{40} was statistically significant ($P < 0.05$). There was no significant difference in the dose-volume relationship of colon, bladder and femoral head between the two groups ($P > 0.05$).

Table 1 Baseline characteristics of all patients (*n* = 50)

Characteristic	*n* (%)
Age, median (IQR)	51.0 (45.5-62.0)
Weight, IQR	55.0 (50.5-61.8)
BMI/kg/m^2, IQR	21.5 (20.7-22.9)
aCCI	
2-3/4-5	38 (76)/12 (24)
Pathological diagnosis	
Squamous cell carcinoma	47 (94)
Adenocarcinoma	1 (2)
Adenosquamous carcinoma	2 (4)
T	
> 4 cm/≤ 4 cm	24 (48)/26 (52)
N	
Negative/positive	15 (30)/35 (70)
Metastatic pelvic lymph nodes	
Positive/negative	14 (28)/36 (72)
Metastatic common iliac lymph nodes	
Positive/negative	5 (10)/45 (90)
Para-aortic lymph nodes	
Positive/negative	2 (4)/48 (96)
FIGO staging	
I/II/III/IVA	19 (38)/21 (42)/5 (10)/5 (10)
LVSI	
Positive/negative	16 (32)/34 (68)
Degree of differentiation	
Low and medium differentiation/high differentiation	40 (80)/10 (20)
Degree of infiltration	
Shallow 1/3/Medium 1/3/Deep 1/3	10 (20)/16 (32)/24 (48)
Incised margin	
R1 + R2/R0	5 (10)/45 (90)
Danger degree	
Low/medium/high risk	15 (30)/12 (24)/23 (46)
HPV	
Positive/negative	46 (92)/4 (8)
SCC	
Abnormally elevated/no abnormality observed	17 (34)/33 (66)
RE	
Occurred/not occurred	42 (84)/8 (16)
SARE/Non-SARE	15 (30)/35 (70)
Operation	
Radical surgery/no surgery	36 (72)/14 (28)
Chemotherapy	

CCRT/SCRT	18 (36)/32 (64)
Pelvic External Radiation Dose, IQR	45.0 (45.0-48.0)
Para-aortic extension field	
Radiotherapy/no radiotherapy	4 (8)/46 (92)

aCCI: Adjusted Charlson Comorbidity Index; SARE: Severe acute radiation enteritis; CCRT: Concurrent chemoradiotherapy; SCRT: Sequential chemoradiotherapy; SCC: Squamous cell carcinoma; HPV: Human papillomavirus.

Table 2 Comparison of general data between postoperative adjuvant radiotherapy and radical radiotherapy for patients with cervical cancer, *n* (%)

Factor	Postoperative adjuvant radiotherapy group	Radical radiotherapy group	T/χ^2 values	*P* value
Age/yr	48.25 ± 8.05	61.86 ± 13.17	-4.450	< 0.001
Young and middle-aged	33 (92)	5 (36)	17.301	< 0.001
Old age	3 (8)	9 (64)		
Weight	57.31 ± 8.00	53.07 ± 8.74	1.638	0.108
BMI/kg/m^2	21.86 ± 2.07	20.21 ± 2.79	2.284	0.027
Tumor type				0.186[1]
Squamous cell carcinoma	35 (97)	12 (86)		
Adenocarcinoma	0 (0)	1 (7)		
Adenosquamous carcinoma	1 (3)	1 (7)		
CCRT			0.397	0.529
Implement	12 (33)	6 (43)		
Not implemented	24 (67)	8 (57)		
Para-aortic extended field radiotherapy				0.186[1]
Implement	1 (3)	2 (14)		
Not implemented	35 (97)	12 (86)		
RE			-	1.000[1]
Occurred	30 (83)	12 (86)		
Did not occur	6 (17)	2 (14)		
SARE			2.286	0.131
Occurred	13 (36)	2 (14)		
Did not occur	23 (64)	12 (86)		

[1]Fisher exact probability method.
Data are shown as *n* (%) or mean ± SD. SARE: Severe acute radiation enteritis; CCRT: Concurrent chemoradiotherapy.

Comparison of the cumulative incidence of VMAT-related acute RE in cervical cancer between the postoperative adjuvant radiotherapy group and the radical radiotherapy group

The main radiotherapy-related adverse reactions included diarrhea, abdominal pain, colitis, anal distension, hematochezia and bone marrow suppression. All adverse reactions were grade 1 to 3; no adverse reactions above grade 3 occurred. RE was monitored as follows (Table 4). There was no significant difference in the incidences of adverse reaction between the radical radiotherapy group and postoperative adjuvant radiotherapy group ($P > 0.05$).

Evaluation of the specificity and sensitivity of OAR dose-volume parameters in the IMRT plan for predicting acute/SARE

ROC curve was used to analyze the specificity and sensitivity of small intestine V_{20}, V_{30}, V_{40} and rectum

Table 3 Organs at risk dose-volume comparison between adjuvant radiotherapy (*n* = 36) and radical radiotherapy (*n* = 14) after cervical cancer surgery (%)

OAR	Group	V_{20}	V_{25}	V_{30}	V_{35}	V_{40}
Small intestine	Postoperative adjuvant radiotherapy group	77.41 ± 16.49	51.90 ± 10.99	39.51 ± 8.24	26.40 ± 5.50	19.25 ± 4.12
	Radical radiotherapy group	67.75 ± 14.10	50.11 ± 9.86	32.92 ± 7.91	25.18 ± 7.12	15.07 ± 4.65
	Z value	-2.377	-0.864	-2.701	-0.367	-3.003
	P value	0.017	0.387	0.007	0.713	0.003
Colon	Postoperative adjuvant radiotherapy group	100.00 ± 0.00	99.88 ± 0.73	98.26 ± 6.09	91.36 ± 11.67	75.64 ± 12.59
	Radical radiotherapy group	95.57 ± 16.59	95.34 ± 17.44	94.21 ± 21.66	91.09 ± 21.12	84.17 ± 7.94
	Z value	-1.604	-0.732	-0.332	-0.750	-2.247
	P value	0.109	0.464	0.740	0.453	0.025
Rectum	Postoperative adjuvant radiotherapy group	100.00 ± 0.00	100.00 ± 0.00	99.17 ± 2.90	95.63 ± 9.41	75.64 ± 12.59
	Radical radiotherapy group	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	99.60 ± 1.48	84.17 ± 7.94
	Z value	0.000	0.000	-1.102	-1.745	-2.247
	P value	1.000	1.000	0.270	0.081	0.025
Bladder	Postoperative adjuvant radiotherapy group	70.87 ± 28.16	68.75 ± 24.41	47.18 ± 23.63	35.58 ± 16.04	27.33 ± 12.22
	Radical radiotherapy group	74.08 ± 27.93	71.94 ± 25.87	52.54 ± 28.93	40.40 ± 21.04	31.18 ± 16.28
	T-value	-0.354	-0.523	-0.519	-0.605	-0.627
	P value	0.723	0.601	0.604	0.545	0.531
Femoral head	Postoperative adjuvant radiotherapy group	48.55 ± 28.80	34.45 ± 22.27	24.73 ± 16.00	18.12 ± 11.68	12.23 ± 8.10
	Radical radiotherapy group	39.60 ± 19.76	27.13 ± 14.03	19.54 ± 10.07	14.42 ± 7.69	9.47 ± 5.37
	T-value	-0.951	-0.929	-0.994	-0.929	-0.983
	P value	0.342	0.353	0.320	0.353	0.326

OAR: Organs at risk.

V_{40} in predicting acute/SARE. The AUC was less than 0.60 and $P > 0.05$. These results suggest that a single OAR dose-volume parameter is not enough to predict the occurrence of acute/SARE.

DAI score changes

The DAI scores of the radical radiotherapy group were higher than those of the postoperative adjuvant radiotherapy group at the second week, fourth week and the end of radiotherapy (Figure 1). There was an interaction effect between different groups and time ($\chi^2 = 77.238$, $P < 0.01$). The DAI scores of the two groups showed an increasing trend over time ($P < 0.05$), and the DAI scores of the radical radiotherapy group increased the most.

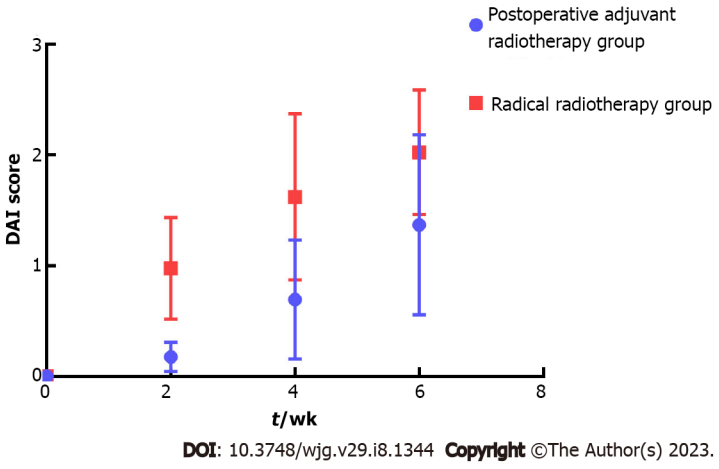
Establishment of the SARE prediction model

Univariate analysis showed that abdominal pain, colitis, anal bulging, hematochezia, DAI score, age, and CCRT were significantly correlated with the occurrence of severe acute RE (all $P < 0.05$) (Table 5). Kendall analysis showed a relatively strong correlation between abdominal pain, hematochezia and DAI score ($R = 0.715$, 0.622, $P < 0.001$); the correlation between other factors was very weak (Figure 2A). To avoid multicollinearity, abdominal pain, hematochezia, and DAI scores were included in the multivariate analysis. Multivariate logistic regression analysis was conducted to analyze the factors of colitis, anal bulge, DAI score and age. In multivariate analysis, anal bulge rating (OR: 14.779, 95%CI: 1.281-170.547, $P = 0.031$) and DAI score (OR: 53.928, 95%CI: 3.822-760.948, $P = 0.003$) were independent predictors of SARE (Table 6). These factors were used to construct the nomogram. The constant in the logistic regression equation was -10.039, and the logistic regression equation was Logit (P) = 3.988 × DAI + 2.693 × anal bulge rating -10.039.

Table 4 Comparison of the incidence of acute radiation enteritis in patients with cervical cancer treated with adjuvant radiotherapy (*n* = 36) and radical radiotherapy (*n* = 14), *n* (%)

Adverse reaction	Group	Level 0	Level 1	Level 2	Level 3	χ^2 values	*P* value
Diarrhea	Postoperative adjuvant radiotherapy group	10 (28)	19 (53)	5 (14)	2 (5)	2.748	0.432
	Radical radiotherapy group	3 (21)	10 (72)	0 (0)	1 (7)		
Abdominal pain	Postoperative adjuvant radiotherapy group	21 (58)	12 (33)	3 (9)	0 (0)	1.423	0.491
	Radical radiotherapy group	8 (57)	6 (43)	0 (0)	0 (0)		
Colitis	Postoperative adjuvant radiotherapy group	25 (69)	11 (31)	0 (0)	0 (0)	-	0.140[1]
	Radical radiotherapy group	13 (93)	1 (7)	0 (0)	0 (0)		
Anal bulging	Postoperative adjuvant radiotherapy group	22 (61)	9 (25)	5 (14)	0 (0)	1.374	0.503
	Radical radiotherapy group	11 (79)	2 (14)	1 (7)	0 (0)		
Blood in the stool	Postoperative adjuvant radiotherapy group	23 (64)	13 (36)	0 (0)	0 (0)		0.179[1]
	Radical radiotherapy group	12 (86)	2 (14)	0 (0)	0 (0)		
Myelosuppression	Postoperative adjuvant radiotherapy group	12 (33)	9 (25)	10 (28)	5 (14)	3.691	0.297
	Radical radiotherapy group	3 (21)	4 (29)	2 (14)	5 (36)		

[1]Fisher exact probability method.



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Figure 1 Disease activity index score during volumetric modulated arc therapy in 50 patients with cervical cancer.

Development and validation of the nomogram

Based on the multivariate logistic regression coefficients, the prediction model was visually represented as a nomogram (Figure 2B). The ROC curves for the anal bulge rating, DAI score and nomogram are shown in Figure 2C. ROC analysis showed that the AUC of the prediction model was 0.950 (95%CI: 0.891-1.000), which was much higher than that of each parameter alone (anal bulge rating: 0.805, 95%CI: 0.651-0.959; DAI score: 0.892, 95%CI: 0.873-1.000). The MAL thresholds for anal bulge rating and DAI score were 0.5 and 2.165 points. The nomogram had a high predictive efficiency (sensitivity: 80.0%, specificity: 91.4%). In addition, the calibration curve showed good agreement between the predicted severe acute RE and the actual observations (Figure 2D). In most threshold probabilities, DCA showed a positive net benefit with a satisfactory nomogram, indicating a good potential clinical effect of the model (Figure 2E).

Table 5 Comparison of general data between patients with cervical cancer with severe acute radiation enteritis or no severe acute radiation enteritis

Factor	SARE (*n* = X)	No SARE (*n* = X)	OR (95%CI)	*P* value
	Median (IQR)	Median (IQR)		
Clinical factors				
Elderly (> 60 yr)/young and middle-aged (20-60 yr)			0.123 (0.015-1.040)	0.054
Age	47.5 (37.0-54.0)	53.0 (46.0-63.0)	0.930 (0.872-0.992)	0.027
Weight	53.0 (51.0-60.9)	56.0 (50.0-61.8)	0.983 (0.918-1.054)	0.631
BMI/kg/m^2	20.5 (19.7-22.2)	21.9 (20.4-23.0)	0.936 (0.746-1.174)	0.566
ACCI (2-3/4-5)			0.123 (0.015-1.040)	0.054
Tumor type (squamous/non-squamous)			0.848 (0.139-5.179)	0.859
T (> 4 cm/≤ 4 cm)			0.510 (0.153-1.695)	0.272
N (positive/negative)			0.615 (0.165-2.298)	0.538
Metastatic pelvic lymph nodes (positive/negative)			0.694 (0.185-2.611)	0.589
Common iliac lymph node metastasis (positive/negative)			0.427 (0.046-3.980)	0.455
Para-aortic lymph nodes (positive/negative)			1.471 (1.216-1.779)	0.999
FIGO stage (IIB-IVA/IB-IIA)			1.108 (0.328-3.739)	0.869
LVSI (positive/negative)			0.548 (0.147-2.034)	0.368
Differentiation degree (low and medium differentiation/high differentiation)			1.011 (0.225-4.536)	0.988
Infiltration degree (shallow 1/3/medium depth 1/3)			0.837 (0.191-3.673)	0.813
Incisal margin (R1 + R2/R0)			0.427 (0.046-3.980)	0.455
Risk (low risk/medium high risk)			1.227 (0.340-4.242)	0.754
HPV (positive/negative)			1.324 (0.127-13.785)	0.815
SCC (abnormally elevated/not abnormal)			0.303 (0.074-1.245)	0.098
Surgery (performed/not performed)			2.080 (0.497-8.706)	0.316
CCRT (implemented/not implemented)			6.042 (1.681-21.718)	0.006
Myelosuppression (Grade 1-3/Grade 0)			0.617 (0.178-2.144)	0.446
Diarrhea (Grade 1-3/Grade 0)			NA	0.999
Abdominal pain (Grade 1-3/Grade 0)			25.375 (4.750-135.559)	< 0.001
Colitis (Grade 1-3/Grade 0)			38.500 (6.530-226.993)	< 0.001
Anal bulge (Grade 1-3/Grade 0)			5.185 (1.470-18.286)	0.010
Hematochezia (Grade 1-3/Grade 0)			NA	< 0.001
DAI score	2.8 (2.3-3.0)	1.7 (1.2-2.0)	152.546 (6.045-3849.771)	0.002
Physical dose parameters				
Small intestine				
V_{20} (%)	80.9 (68.6-93.7)	78.0 (66.8-82.5)	1.015 (0.975-1.056)	0.475
V_{25} (%)	58.2 (46.0-62.9)	52.0 (48.0-57.3)	1.014 (0.955-1.076)	0.659
V_{30} (%)	41.0 (35.1-47.6)	39.6 (31.1-42.1)	1.039 (0.961-1.122)	0.335
V_{35} (%)	29.6 (23.5-32.2)	26.9 (24.4-29.1)	1.032 (0.927-1.149)	0.569
V_{40} (%)	19.5 (16.8-23.3)	19.3 (13.3-20.7)	1.068 (0.928-1.229)	0.358
Rectum				
V_{20} (%)	80.9 (68.6-93.7)	78.0 (66.8-82.5)	1.015 (0.975-1.056)	0.475

V_{25} (%)	58.2 (46.0-62.9)	52.0 (48.0-57.3)	1.014 (0.955-1.076)	0.659
V_{30} (%)	41.0 (35.1-47.6)	39.6 (31.1-42.1)	1.039 (0.961-1.122)	0.335
V_{35} (%)	29.6 (23.5-32.2)	26.9 (24.4-29.1)	1.032 (0.927-1.149)	0.569
V_{40} (%)	19.5 (16.8-23.3)	19.3 (13.3-20.7)	1.068 (0.928-1.229)	0.358
Colon				
V_{20} (%)	100.0 (100.0-100.0)	100.0 (100.0-100.0)	NA	NA
V_{25} (%)	100.0 (100.0-100.0)	100.0 (100.0-100.0)	NA	NA
V_{30} (%)	100.0 (100.0-100.0)	100.0 (100.0-100.0)	0.982 (0.775-1.246)	0.883
V_{35} (%)	100.0 (99.1-100.0)	100.0 (100.0-100.0)	0.981 (0.915-1.052)	0.981
V_{40} (%)	80.4 (71.3-89.2)	79.3 (72.1-87.8)	1.005 (0.956-1.056)	0.851
Femoral head				
V_{20} (%)	35.4 (19.7-57.3)	48.6 (22.9-65.2)	0.994 (0.971-1.017)	0.586
V_{25} (%)	24.8 (13.7-40.1)	31.6 (16.0-45.1)	0.996 (0.967-1.026)	0.797
V_{30} (%)	17.7 (9.8-29.6)	22.6 (11.4-32.6)	0.997 (0.957-1.039)	0.877
V_{35} (%)	13.3 (7.4-20.0)	16.9 (8.6-24.5)	0.989 (0.935-1.047)	0.711
V_{40} (%)	8.9 (4.9-14.3)	11.3 (5.7-16.3)	0.993 (0.916-1.076)	0.858
Total pelvic lymphatic drainage dose (Gy)	45.0 (45.0-45.0)	45.0 (45.0-48.5)	1.194 (0.877-1.625)	0.261
Para-aortic extended field radiotherapy (performed/not performed)			1.485 (1.222-1.804)	0.999

ACCI: Adjusted Charlson Comorbidity Index; CCRT: Concurrent chemoradiotherapy; SCC: Squamous cell carcinoma; HPV: Human papillomavirus; NA: Not available; DAI: Disease activity index.

Table 6 Multivariate analysis of influencing factors of severe radiation enteritis in 50 patients with cervical cancer treated with radiotherapy

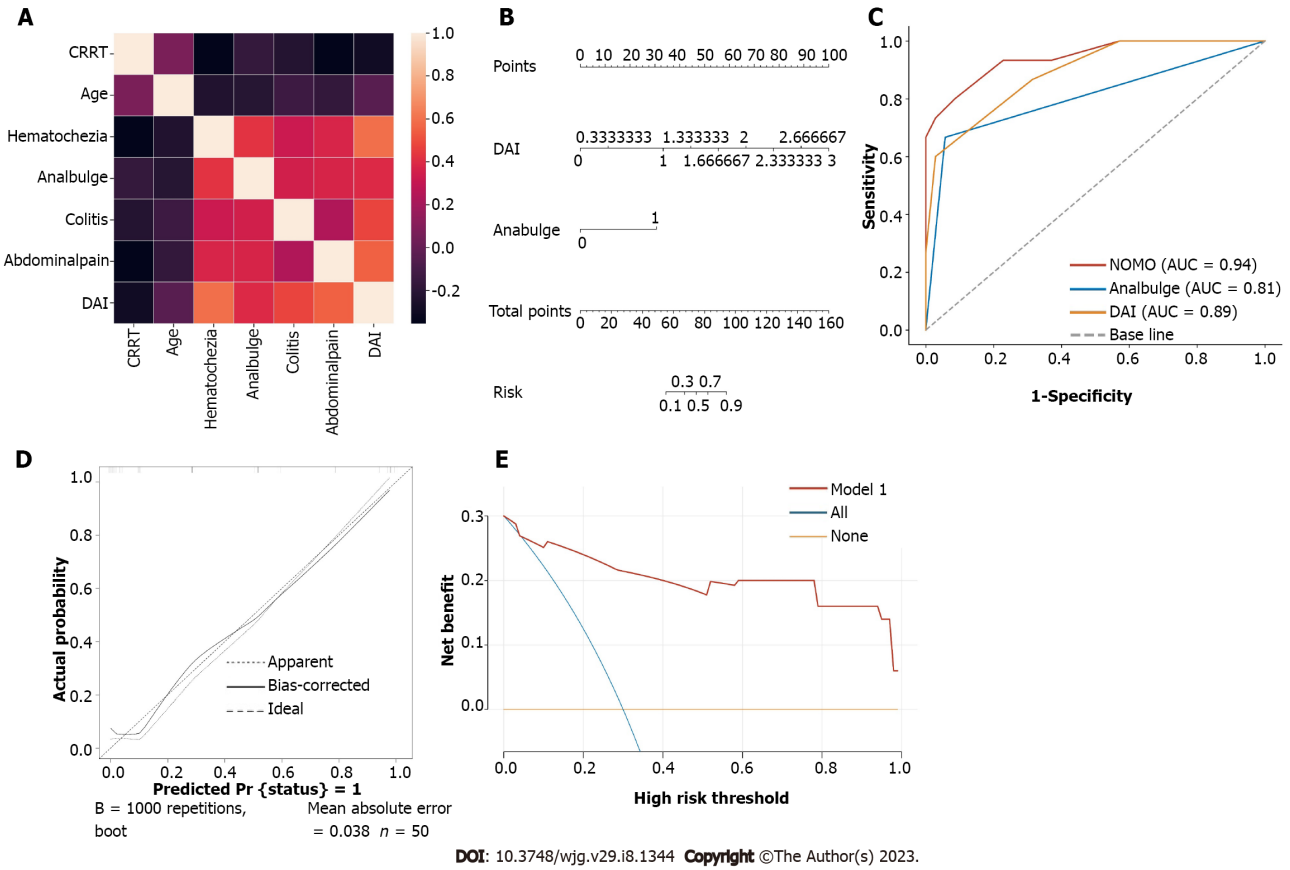
	B	SE	Exp (B)	95%CI		Wald χ^2	*P* value
				Lower	Upper		
DAI score	4.106	1.371	60.705	4.134	891.506	9.463	0.003
Anal bulge rating	2.925	1.229	18.636	1.677	207.114	7.664	0.017

DAI: Disease activity index; 95%CI: 95% confidence interval.

DISCUSSION

Nearly 80% of patients receiving pelvic radiotherapy for cervical cancer have early acute toxicity and 20% have late toxicity[16]. Acute RE often occurs in the second week after the start of radiotherapy and reaches a peak in the fourth to fifth week. The clinical manifestations are nausea, vomiting, abdominal pain, bloody stool, mucous stool, diarrhea, tenesmus, and incontinence. In gynecological tumor radiotherapy, the clinical application of IMRT shows an increasing trend. This radiotherapy technology can not only obtain satisfactory target coverage dose, but also effectively reduces the radiation dose of OAR[17,18], such as V45 of rectum and small intestine[19,20], and reduces the toxic reaction of bone marrow[21,22]. Although the incidence and severity of RE have decreased under the current radiotherapy technology, these issues cannot be ignored. In addition, mild RE is often underestimated or ignored due to the lack of clinical records and inadequate assessment of toxicity[23].

In this study, we preliminarily demonstrated the close relationship between the anal bulge rating and the DAI score and SARE. More importantly, this study is the first to develop and validate an easy-to-understand visual nomogram prediction model for cervical cancer radiotherapy patients and doctors, providing more personalized and accurate SARE prediction for cervical cancer patients undergoing radiotherapy. Many studies have reported that the most important factor affecting radiation proctitis is the total dose received by the rectum, and it is also affected by the radiotherapy technique, fraction dose, total dose received by the rectum/total volume, dose volume ratio of rectum to radiation dose,



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Figure 2 Development and validation of the nomogram. A: Kendall's rank correlation analyses among factors with $P < 0.05$ in univariate logistic regression; B: Nomogram predicting the occurrence of severe acute radiation enteritis (SARE). For each individual patient, two lines are drawn upward to determine the points received from the two variables in the nomogram; the sum of these points is located on the "Total Points" line, and a line is drawn downward to determine the likelihood of this patient to have SARE; C: Receiver operating characteristic curves of anal bulge rating. Disease activity index score *vs* the predictive model; D: Calibration curves of the nomogram predicting the occurrence of SARE. The x-axis and y-axis indicate the predicted and actual probabilities of having SARE, respectively; E: Decision curves of the nomogram predicting the occurrence of SARE. The x-axis shows the threshold probabilities. The y-axis measures the net benefit, which was calculated by adding the true positives and subtracting the false positives. NOMO: Nodal modulator; AUC: Area under the receiver operating characteristic curve; DAI: Disease activity index.

and uniformity of radiation dose distribution[4-6]. According to Seppenwoolde *et al*[24], rectal V40 ≥ 80% is a physical dose factor predicting VMAT-related rectal incontinence and diarrhea in patients with locally advanced cervical cancer. Ballari *et al*[25] suggested that small bowel V45 has predictive value for acute RE. In addition, individual factors such as IBD, collagen vascular disease, history of abdominal and pelvic surgery, history of pelvic inflammatory disease, diabetes mellitus, and acquired immunodeficiency syndrome affect the determination of radiation dose and volume[7-11]. In patients with cervical cancer, CCRT exerts radiosensitization and increases gastrointestinal toxicity by a factor of 3[18]. However, there is currently no consensus on the relative importance of these relevant predictors. In our study, the dose-volume of the small intestine receiving 20-40 Gy in the radical radiotherapy group of cervical cancer was significantly reduced compared with that in the postoperative adjuvant radiotherapy group, and the reduction of V_{20}, V_{30}, and V_{40} was statistically significant ($P < 0.05$), which was considered to be related to the anatomical displacement of organs after hysterectomy. After the removal of the tumor bed and uterus, part of the intestinal loop falls into the pelvic cavity[9], resulting in increased secondary toxicity of radiotherapy. In addition, the rectal V_{30}, V_{35}, and V_{40} in the radical radiotherapy group were significantly higher than those in the postoperative adjuvant radiotherapy group, and the increase of V_{40} was statistically significant ($P < 0.05$). The cervical cancer patients treated with radical radiotherapy in this study were FIGO IIB-IVA. CT or MRI showed obvious parametrial infiltration, and mesorectal invasion occurred in some patients, resulting in a relative increase in the volume of PTV to the left, right and dorsal side. Although we found dose-volume differences in the small intestine and rectum between groups, we were unable to demonstrate that a single physical parameter independently predicted RE or SARE. Therefore, more prospective, well-designed randomized controlled trials with larger sample sizes are needed for validation.

Acute RE is self-limited, and not all acute RE will be classified as chronic RE. The prevalence of chronic RE after 10 years of radiotherapy is 10%-20%[26]. In terms of pathogenesis, inflammation and cell death occur in the intestinal mucosa during the acute phase of RE, and sustained cytokine activation

in the submucosa leads to progressive ischemia, fibrosis, and stem cell loss[27]. In contrast, chronic RE is associated with progressive endarteritis obliterans, secondary to capillary fibrosis, leading to chronic ischemia and fibrosis of the affected bowel segments[28]. The outcome of chronic RE often induces intestinal obstruction, which is similar to the Koenig syndrome observed in Crohn's disease and is considered to be related to ileal stenosis. In view of the similarities between RE and IBD, we innovatively adopted the IBD evaluation index-DAI score. Univariate and multivariate logistic regression analysis confirmed that DAI score was independent of other physical parameters and clinical factors and could be used as a new predictor of SARE. In univariate analysis, abdominal pain, colitis, anal distension, hematochezia, DAI score, age, and CCRT were statistically correlated with the occurrence of severe acute RE ($P < 0.05$). Kendall analysis showed a relatively strong correlation between abdominal pain, hematochezia, and DAI scores. Therefore, DAI score is a convenient, non-invasive and highly beneficial diagnostic system for SARE.

The clinical manifestations of SARE are complex, and it is difficult for doctors, even with extensive clinical experience, to predict and diagnose the disease early, comprehensively and accurately. If cervical cancer can be identified and treated early before, during or after radiotherapy, the toxic and side effects of pelvic radiotherapy may be improved, and the probability of radiotherapy intolerance or even termination of radiotherapy may be reduced. The multi-factor prediction model is helpful to further improve the prediction of RE. Several prediction models have been described in previous studies, but they are still not used in clinic due to the low recognition ability of RE and the neglect of heterogeneity among different patients[29,30]. In this study, we established a SARE-SS evaluation system and found that anal bulge rating and DAI score were independent predictors of SARE through multivariate statistical analysis. The above predictors were further integrated into the nomogram to allow precise individualized SARE risk assessment for each patient. The AUC of the predictive model was 0.950, which was much higher than the two independent factors of DAI score and anal bulge score. The MAL thresholds for anal bulge rating and DAI score were 0.5 grade and 2.165 points, respectively, indicating an advantage over previous models in predicting RE. We adopted the Bootstrap validation method (including drawing the calibration curve and DCA curve), which showed a statistical advantage in a relatively small sample size statistic[31,32]. In later studies, we will expand the sample size, carry out prospective cohort studies, and use external validation methods to reduce data selection bias and increase test power.

Although our study has many advantages, there are still several limitations to be addressed. First, our sample size is small, so a large cohort is needed to further develop and validate the nomogram for predicting RE. Second, since this was a retrospective study, our study may have had a selection bias. Third, we only integrated the most important and commonly used parameters in clinic. From the perspective of precision medicine, we need to combine biological factors in the future including individual genomics, proteomics, metabolomics, microbiomics, real-time dosimetry and a wider range of clinical parameters to establish a comprehensive prediction model to improve the accuracy of RE prediction. Therefore, we will add biological factors to the analysis of related studies in later studies and need to establish a more comprehensive and specific prediction model of RE.

CONCLUSION

Our findings showed that during VMAT for cervical cancer, gastrointestinal and hematological system toxicity gradually increased with radiotherapy treatment and reached the peak at the end of radiotherapy. The main adverse reactions were diarrhea, abdominal pain, colitis, anal bulging and hematochezia. There were significant differences in V_{20}, V_{30}, V_{40} of small intestine and V_{40} of rectum between postoperative adjuvant radiotherapy and radical radiotherapy, which may be related to intestinal anatomical displacement caused by surgery and primary disease. However, there were no significant differences in the cumulative incidence of adverse reactions between the two groups. Anal bulge rating (> 0.500) and DAI score (> 2.165) were identified as independent predictors of SARE by multivariate analysis. The validity of our established nomogram was verified by the internal verification method. Although further external validation is needed, our results indicate that the nomogram has clinical value for the prediction and evaluation of SARE.

ARTICLE HIGHLIGHTS

Research background

Radiation enteritis (RE) not only seriously affects the quality of life of patients, but it also leads to radiotherapy intolerance or termination of radiotherapy. However, data on the clinical efficacy and side effects of volumetric modulated arc therapy (VMAT) for cervical cancer are limited.

Research motivation

If the incidence of RE in patients can be predicted in advance, and targeted clinical preventive treatment can be carried out, the side effects of radiotherapy in cervical cancer patients can be significantly reduced. Furthermore, accurate prediction of RE is essential for the selection of individualized radiation dose and the optimization of the radiotherapy plan.

Research objectives

To analyze the relationships between severe acute RE (SARE) of cervical cancer radiotherapy and clinical factors and dose-volume parameters retrospectively.

Research methods

We included 50 cervical cancer patients who received VMAT from September 2017 to June 2018 in the Department of Radiotherapy at The First Affiliated Hospital Soochow University. Clinical and dose-volume histogram factors of patients were collected. Logistic regression analysis was used to evaluate the predictive value of each factor for SARE. A nomogram to predict SARE was developed (SARE scoring system ≥ 3 points) based on the multiple regression coefficients; validity was verified by an internal verification method.

Research results

Gastrointestinal and hematological toxicity of cervical cancer VMAT gradually increased with radiotherapy and reached the peak at the end of radiotherapy. The main adverse reactions were diarrhea, abdominal pain, colitis, anal swelling, and blood in the stool. There was no significant difference in the incidence of gastrointestinal toxicity between the radical and postoperative adjuvant radiotherapy groups ($P > 0.05$). There were significant differences in the small intestine V20, V30, V40, and rectal V40 between adjuvant radiotherapy and radical radiotherapy after surgery ($P < 0.05$). Univariate and multivariate analysis revealed anal bulge rating (OR: 14.779, 95%CI: 1.281-170.547, $P = 0.031$) and disease activity index (DAI) score (OR: 53.928, 95%CI: 3.822-760.948, $P = 0.003$) as independent predictors of SARE.

Research conclusions

Anal bulge rating (> 0.500 grade) and DAI score (> 2.165 points) can predict SARE. The nomogram shows potential value in clinical practice.

Research perspectives

From the perspective of precision medicine, it will also be necessary to combine biological factors, such as individual genomics, proteomics, metabolomics, microbiomics, real-time dosimetry, and a wider range of clinical parameters to establish comprehensive predictive models. This study is a prospective study with a small sample size. In the later stage, we will expand the sample size, conduct prospective cohort studies, and use external validation methods to reduce data selection bias and increase test efficiency.

FOOTNOTES

Author contributions: Ma CY and Zhao J contributed to conceptualization and methodology; He XL and Gan GH contributed to data curation; Ma CY contributed to formal analysis; Ma CY, Zhou JY contributed to funding acquisition; Zhou JY, Xu XT, Qin SB contributed to project administration; Zhou JY, Xu XT, Qin SB, Wang LL, and Li L contributed to resources; Xu XT and Qin SB contributed to supervision; Ma CY contributed to validation and visualization; Ma CY and Zhao J contributed to roles/writing-original draft; Ma CY contributed to writing-review and editing.

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Data sharing statement: The datasets generated and analyzed during the present study are available from the corresponding author on reasonable request.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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Country/Territory of origin: China

ORCID number: Ju-Ying Zhou 0000-0002-2796-6388.

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ORIGINAL ARTICLE

Observational Study

Better performance of PIVKA-II for detecting hepatocellular carcinoma in patients with chronic liver disease with normal total bilirubin

Xiang-Jun Qian, Zhu-Mei Wen, Xiao-Ming Huang, Hui-Juan Feng, Shan-Shan Lin, Yan-Na Liu, Sheng-Cong Li, Yu Zhang, Wen-Guang Peng, Jia-Rui Yang, Zhe-Yu Zheng, Lei Zhang, Da-Wei Zhang, Feng-Min Lu, Li-Juan Liu, Wei-Dong Pan

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Xiang-Jun Qian, Xiao-Ming Huang, Wen-Guang Peng, Jia-Rui Yang, Zhe-Yu Zheng, Lei Zhang, Da-Wei Zhang, Wei-Dong Pan, Department of Pancreatic Hepatobiliary Surgery, The Sixth Affiliated Hospital of Sun Yat-sen University, Guangzhou 510655, Guangdong Province, China

Xiang-Jun Qian, Yan-Na Liu, Feng-Min Lu, Department of Microbiology & Infectious Disease Center, School of Basic Medical Sciences, Peking University Health Science Center, Beijing 100191, China

Zhu-Mei Wen, Hui-Juan Feng, Sheng-Cong Li, Yu Zhang, Li-Juan Liu, Department of Laboratory Medicine, Mengchao Hepatobiliary Hospital of Fujian Medical University, Fuzhou 350025, Fujian Province, China

Shan-Shan Lin, Johns Hopkins Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD 21205, United States

Corresponding author: Wei-Dong Pan, MD, Doctor, Professor, Surgeon, Department of Pancreatic Hepatobiliary Surgery, The Sixth Affiliated Hospital of Sun Yat-sen University, No. 26 Erheng Road, Tianhe District, Guangzhou 510655, Guangdong Province, China.
panwd@mail.sysu.edu.cn

Abstract

BACKGROUND

Serum protein induced by vitamin K absence or antagonist-II (PIVKA-II) is a promising biomarker for hepatocellular carcinoma (HCC) surveillance.

AIM

To identify the contributing factors related to the abnormal elevation of PIVKA-II level and assess their potential influence on the performance of PIVKA-II in detecting HCC.

METHODS

This study retrospectively enrolled in 784 chronic liver disease (CLD) patients and 267 HCC patients in Mengchao Hepatobiliary Hospital of Fujian Medical University from April 2016 to December 2019. Logistic regression and the area

under the receiver operating characteristic curve (AUC) were used to evaluate the influencing factors and diagnostic performance of PIVKA-II for HCC, respectively.

RESULTS

Elevated PIVKA-II levels were independently positively associated with alcohol-related liver disease, serum alkaline phosphatase (ALP), and total bilirubin (TBIL) for CLD patients and aspartate aminotransferase (AST) and tumor size for HCC patients (all $P < 0.05$). Serum PIVKA-II were significantly lower in patients with viral etiology, ALP ≤ 1 × upper limit of normal (ULN), TBIL ≤ 1 × ULN, and AST ≤ 1 × ULN than in those with nonviral disease and abnormal ALP, TBIL, or AST (all $P < 0.05$), but the differences disappeared in patients with early-stage HCC. For patients with TBIL ≤ 1 × ULN, the AUC of PIVKA-II was significantly higher compared to that in patients with TBIL > 1 × ULN (0.817 *vs* 0.669, $P = 0.015$), while the difference between ALP ≤ 1 × ULN and ALP > 1 × ULN was not statistically significant (0.783 *vs* 0.729, $P = 0.398$). These trends were then more prominently perceived in subgroups of patients with viral etiology and HBV alone.

CONCLUSION

Serum PIVKA-II has better performance in detecting HCC at an early stage for CLD patients with normal serum TBIL.

Key Words: Protein induced by vitamin K absence or antagonist-II; Chronic liver disease; Total bilirubin; Hepatocellular carcinoma; Diagnosis; Hepatitis B virus

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Core Tip: This study demonstrated that elevated serum protein induced by vitamin K absence or antagonist-II (PIVKA-II) were positively associated with serum total bilirubin (TBIL) in patients with chronic liver disease (CLD), and the levels of PIVKA-II in CLD patients with normal serum TBIL were significantly lower than those in CLD patients with abnormal serum TBIL. Serum PIVKA-II has better performance in detecting hepatocellular carcinoma (HCC) at an early stage for CLD patients with normal serum TBIL, which was more prominently perceived in patients with viral etiology and hepatitis B virus alone. These findings may be important for surveillance counseling of early-stage HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC), a serious issue for global health, is still the sixth most prevalently diagnosed cancer and the third highest cause of cancer death worldwide[1,2]. Chronic liver disease (CLD) induced by viral and nonviral factors has been identified as the main cause of HCC, including chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), alcohol-related liver disease (ALD), nonalcoholic fatty liver disease (NAFLD), autoimmune liver disease (AILD), and so on, with the major risk factors varying from region to region[1-3].

In recent years, although great progress has been achieved on cancer treatment with the development by surgical resection, molecular targeted therapy, and immunotherapy[2,4-6], 5-year survival rate of HCC remains not satisfactory, with only 15%-17% as reported[7,8]. The main cause of this extremely poor prognosis is that more than half of HCC patients present with advanced stages once at diagnosis[7,8]. Detecting HCC at an early stage is an efficacious way for the improvement of long term survival of HCC patients[2,9,10].

Serum HCC biomarkers as surveillance tools remain controversial because the diagnostic accuracy of the traditional biomarker for HCC, serum alpha-fetoprotein (AFP), is unsatisfactory[8,11,12]. Serum AFP is frequently influenced by many noncancerous factors and is falsely raised in non-HCC patients who have active chronic viral and advanced liver disease[13-15]. Hence, protein induced by vitamin K absence or antagonist-II (PIVKA-II), has gained increasing attention in recent years since it was first

reported for the diagnosis of HCC in 1984[2,8,9,16]. PIVKA-II is poorly related to AFP, and the diagnostic performance of HCC by combining the two biomarkers was higher compared to AFP alone[8, 17]. In addition, the guidelines of the Japan Society of Hepatology has recommended serum PIVKA-II as one of the HCC surveillance markers in at-risk populations[18,19].

PIVKA-II could also be employed to monitor HCC prognosis, treatment response, and recurrence because of its possible associations with the tumor size, HCC progression and recurrence[20,21]. Serum PIVKA-II levels have also been reported to be influenced by several noncancerous factors, such as vitamin K deficiency[22], ALD[23-26], acute hepatic failure[27], and the use of warfarin or antibiotics[26, 28] by leading to serum PIVKA-II elevation in non-HCC patients.

However, it remains unclear what the factors are regarding different liver disease etiologies, the liver injury severity, and disease activity degree associated with abnormal PIVKA-II levels in patients with CLD and HCC. Therefore, we aimed to identify the potential contributing factors and evaluate their influence on serum PIVKA-II levels and the performance of PIVKA-II for the diagnosis of HCC in different populations.

MATERIALS AND METHODS

Patients selection

This study eventually retrospectively registered 1051 patients after excluding 80 patients from 1131 registered patients. The mean age of the total cohort was 48.93 years ± 13.70 years, and 73.6% of them were male (n = 773). Supplementary Figure 1 shows the selection flowchart and the analysis process. All patients were recruited in Mengchao Hepatobiliary Hospital of Fujian Medical University from April 2016 to December 2019.

The patients included following the criteria as: (1) With CLD or HCC of clear etiology diagnosed by clinical or histological evidence, including chronic hepatitis B (CHB), chronic hepatitis C (CHC), ALD, NAFLD, and AILD; (2) CHB and CHC were defined as patients with positive hepatitis B surface antigen (HBsAg) and positive hepatitis C virus (HCV) antibodies and/or HCV RNA for at least 6 months; and (3) complete and detailed laboratory and clinical information, including serum PIVKA-II, relevant laboratory tests, and other clearly clinical characteristics and records. The patients excluded following the criteria as: (1) Patients diagnosed with CHB and ALD simultaneously; (2) patients with liver failure, esophageal and gastric variceal bleeding, or drug-induced liver disease; (3) patients without sufficient information on relevant laboratory tests and clinical records; (4) patients without a certain clinical diagnosis of HCC; (5) patients accepting warfarin or long-term treatment with antibiotics at the time of enrollment; and (6) patients with other malignant tumors or a prior history of antitumor treatment in HCC.

According to the Milan criteria, early-stage HCC is defined as a nodule with the diameter less than 5 cm or two to three nodules with the diameter each less than 3 cm and do not have significant vascular invasion or extrahepatic metastases[2,15,29], and beyond the Milan criteria was considered as late-stage HCC. A positive lesion discovered by the suggested imaging techniques and contrast agents or histopathological confirmation were used to determine the diagnosis of HCC[2,3,9,15,30]. For CLD patients at enrollment, the lack of an HCC was determined through that there were no any suspicious hepatic masses in clinical and imaging evidence, and if with an abnormal PIVKA-II level, a continuous imaging surveillance within the subsequent months was appropriate.

The existence of cirrhosis was generally determined *via* imaging, laboratory and clinical characteristics, or liver biopsy without a routine examination.

The ethics committee of Peking University Health Science Center granted this study approval (IRB00001052-19081). All procedures carried out in this study had followed the Helsinki declaration or equivalent ethical principles.

Study variables

Serum PIVKA-II level was quantitatively detected through the chemiluminescence enzyme immunoassay (LUMIPULSE® G1200, FUJIREBIO INC, Japan) by using Lumipulse® G PIVKA-II reaction cartridges in the clinical laboratory of Mengchao Hepatobiliary Hospital of Fujian Medical University, according to manufacturer's instructions. The lower limit of detection is > 0 mAU/mL, and the upper is 75000 mAU/mL. The upper limit of normal (ULN) was 40 mAU/mL[9]. Furthermore, abnormal elevation of serum PIVKA-II was defined as its values elevated above 40 mAU/mL (ULN).

Liver-related laboratory tests, including liver enzyme and function and routine blood, were performed in the clinical laboratory using commercially available kits by manufacturers' instructions. Test values would be reviewed if judged as unusual in order to ensure their accuracy.

Statistical analysis

SPSS 24.0 software (New York, NK, United States) was applied to finish the basic statistical analyses. The mean ± SD or median and interquartile range (IQR), as the continuous variables, were analyzed by t or Mann-Whitney test between two groups or Kruskal-Wallis test among three or more groups; χ^2 test

for categorical variables. Univariate and multivariate (forward) analyses of logistic regression were constructed to investigate factors associated with the abnormal elevation of serum PIVKA-II for non-HCC and HCC patients, respectively. Then, GraphPad Prism 7 (California, CA, United States) was used to plot the distributions of the levels of PIVKA-II in different subgroups and further compare them. The area under receiver operating characteristic (ROC) curve (AUC) and 95% confidence interval (CI), which reflecting the PIVKA-II's performance for HCC, were conducted and calculated by MedCalc version 18.2.1 (Ostend, Belgium). The best cutoff value, sensitivity, specificity, positive likelihood ratio (LR+), and negative likelihood ratio (LR-) of PIVKA-II levels were also calculated and shown. $P < 0.05$ was deemed statistically significant, and all tests and power analyses of significance were two-tailed.

RESULTS

Patient characteristics

The comprehensive clinical and laboratory characteristics of the study population are listed in Table 1. A total of 595 viral liver disease patients, 189 nonviral liver disease patients, 127 early-stage HCC patients, and 140 late-stage HCC patients were enrolled in. Compared to viral liver disease patients, patients with nonviral liver disease had significantly older mean age and higher median serum platelet (PLT) and GGT levels ($P < 0.05$); however, their male proportion and median serum ALT, AST and TBIL levels were significantly lower ($P < 0.05$). Interestingly, the abnormally elevated proportion of PIVKA-II > 40 mAU/mL (ULN) in patients with nonviral liver disease was significantly higher than that in patients with viral liver disease (20.6% *vs* 13.9%, $P < 0.050$), as well as the substantially higher median levels of serum PIVKA-II (27.0 *vs* 23.0, $P < 0.001$).

The median serum ALT, AST, ALP, GGT, and PLT levels in patients with early-stage HCC were significantly lower compared to those in patients with late-stage HCC ($P < 0.01$); and the significantly smaller tumor size and proportion of the number of tumors ≥ 2 were also observed in early-stage HCC patients (both $P < 0.001$), but they had a higher median level of serum ALB ($P < 0.05$). Furthermore, patients with early-stage HCC had a significantly lower abnormal proportion of PIVKA-II > 40 mAU/mL (ULN) than patients with late-stage HCC (60.6% *vs* 93.6%, $P < 0.001$), as well as significantly lower median serum PIVKA-II level (58 *vs* 5124, $P < 0.001$).

Identify the independent factors related to the abnormal elevation of PIVKA-II level (> 1 × ULN) in non-HCC and HCC patients

Then, we further analyzed and identified the independent factors related to the abnormal elevation of PIVKA-II level in non-HCC and HCC patients through logistic regression analyses, respectively. Table 2 and Figure 1A summarize the results of non-HCC patients. ALD etiology (OR: 9.883, 95%CI: 2.216-44.086, $P < 0.001$) was an independent positive factor associated with the abnormal elevation of serum PIVKA-II in non-HCC patients, as well as the factors of ALP/ULN (OR: 2.146, 95%CI: 1.429-3.221, $P < 0.001$) and TBIL/ULN (OR: 1.162, 95%CI: 1.080-1.250, $P < 0.001$). Table 3 and Figure 1B summarize the results of HCC patients. AST/ULN (OR: 1.759, 95%CI: 1.072-2.887, $P = 0.025$) was an independent positive factor associated with the abnormal elevation of serum PIVKA-II in HCC patients, as well as the factor of tumor size (OR: 1.349, 95%CI: 1.175-1.549, $P < 0.001$). These findings implied that there were obvious differences in the factors associated with the abnormal elevation of PIVKA-II level between non-HCC and HCC patients.

Serum PIVKA-II levels were different among different subgroups of patients with non-HCC, early-stage HCC, and late-stage HCC

The distributions and comparisons of serum PIVKA-II were further investigated according to the above results of independent factors associated with the abnormal elevation of PIVKA-II level.

According to Figure 2A, the median PIVKA-II levels among subgroups of different etiologies were significantly different ($P < 0.0001$); for viral liver disease without HCC, no difference was observed between HBV and HCV (23.8 *vs* 21.5, $P > 0.05$), but among nonviral liver diseases of NAFLD, ALD and AILD (28.0 *vs* 34.5 *vs* 20.0, $P < 0.0001$), the differences were significant. Further analysis showed that compared to CLD patients with HBV or HCV, the median PIVKA-II level in ALD was substantially higher (both $P < 0.0001$), and also in NAFLD (both $P < 0.05$). Meanwhile, AILD patients had significantly lower PIVKA-II levels than HBV patients ($P < 0.05$), whereas no difference was observed between HCV and AILD ($P > 0.05$). For patients with HCC (Figure 2B), the median PIVKA-II levels gradually significantly increased as the tumor size changed by ≤ 2 cm, > 2 cm and ≤ 5 cm, > 5 cm and ≤ 10 cm to > 10 cm (39.5 *vs* 135.5 *vs* 1811.0 *vs* 30987.0, $P < 0.0001$).

Further analysis revealed that viral liver disease patients had a significantly lower median level of PIVKA-II than patients with nonviral liver disease (23.0 *vs* 27.0, $P < 0.0001$) in non-HCC (Figure 2C), and there were also similar trends between ALP ≤ 1 × ULN and ALP > 1 × ULN (23.0 *vs* 30.0, $P < 0.0001$) (Figure 2D), TBIL ≤ 1 × ULN and TBIL > 1 × ULN (23.0 *vs* 27.0, $P < 0.0001$) (Figure 2E), AST ≤ 1 × ULN and AST > 1 × ULN (23.0 *vs* 26.0, $P < 0.01$) (Figure 2F) in non-HCC. However, similar tendencies were

Table 1 Clinical characteristics of patients with non-hepatocellular carcinoma and hepatocellular carcinoma

Variable	Non-HCC			HCC		
	Viral liver disease (*n* = 595)	Nonviral liver disease (*n* = 189)	*P* value	Early stage HCC (*n* = 127)	Late stage HCC (*n* = 140)	*P* value
Age (yr)	46.2 ± 13.4	49.6 ± 13.5	0.003	56.5 ± 10.8	55.5 ± 12.1	0.488
Male, *n* (%)	418 (70.3)	117 (61.9)	0.032	113 (89.0)	125 (89.3)	0.935
Etiology			-			0.333
HBV	561 (94.3)	-		115 (90.6)	130 (92.9)	
HCV	34 (5.7)	-		2 (1.6)	2 (1.4)	
NAFLD	-	56 (29.6)		2 (1.6)	-	
ALD	-	82 (43.4)		6 (4.7)	8 (5.7)	
AILD	-	51 (27.0)		2 (1.6)	-	
Cirrhosis, *n* (%)	274 (46.1)	75 (39.7)	0.125	112 (88.2)	120 (85.7)	0.550
ALT (U/L)	48 (25, 171)	40 (24, 74)	< 0.001	31 (22, 42)	40 (25, 67)	0.003
AST (U/L)	44 (27, 101)	38 (25, 60)	0.007	32 (24, 47)	60 (35, 95)	< 0.001
ALP (U/L)	92 (75, 122)	97 (72, 141)	0.153	90 (70, 120)	112 (88, 177)	< 0.001
GGT (U/L)	50 (28, 113)	94 (39, 241)	< 0.001	44 (25, 78)	113 (57, 208)	< 0.001
TBIL (μmol/L)	19.8 (13.2, 31.6)	16.1 (10.5, 35.0)	0.006	18.4 (11.6, 29.4)	19.3 (13.6, 30.2)	0.277
ALB (g/L)	38.8 ± 6.3	38.7 ± 7.7	0.834	38.3 ± 7.0	36.6 ± 6.0	0.036
PLT (10^9/L)	161 (104, 199)	206 (124, 273)	< 0.001	145 (87, 178)	188 (132, 242)	< 0.001
Tumor size (cm)	-	-	-	2.2 (1.5, 2.9)	9.3 (5.8, 13.2)	< 0.001
Number of tumors (1/2-3/> 3)	-	-	-	115/12/0	62/33/45	< 0.001
Vascular invasion	-	-	-	-	53 (37.9)	-
Extrahepatic metastases	-	-	-	-	13 (9.3)	-
PIVKA II > 40 mAU/mL, *n* (%)	83 (13.9)	39 (20.6)	0.027	77 (60.6)	131 (93.6)	< 0.001
PIVKA II (mAU/mL)	23.0 (18.0, 31.0)	27.0 (20.0, 38.0)	< 0.001	58.0 (25.0, 228.0)	5124 (691.0, 36245.0)	< 0.001

HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NAFLD: Nonalcoholic fatty liver disease; ALD: Alcohol-related liver disease; AILD: Autoimmune liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyltranspeptidase; TBIL: Total bilirubin; ALB: Albumin; PLT: Platelet; PIVKA-II: Protein induced by vitamin K absence or antagonist-II.

not observed in early-stage HCC patients, and the PIVKA-II levels between the above subgroups did not differ significantly. Furthermore, for late-stage HCC, the median level of PIVKA-II of ALP ≤ 1 × ULN was significantly lower compared to that of ALP > 1 × ULN (2129 *vs* 11992, $P < 0.01$), and also between AST ≤ 1 × ULN and AST > 1 × ULN (593.0 *vs* 15164, $P < 0.0001$); however, between viral and nonviral liver disease, a significant difference was not observed, nor between TBIL ≤ 1 × ULN and TBIL > 1 × ULN.

Additionally, Supplementary Table 1 shows that irrespective of the subgroups of etiology (viral and nonviral liver disease), ALP (≤ 1 × ULN and > 1 × ULN), TBIL (≤ 1 × ULN and > 1 × ULN), AST (≤ 1 × ULN and > 1 × ULN), serum PIVKA-II levels differed significantly among non-HCC, early-stage and late-stage HCC.

Significant influence of serum TBIL on the performance of PIVKA-II in diagnosing early-stage HCC but not for late-stage HCC

The above findings suggested that abnormally elevated PIVKA-II level was independently positively associated with etiology, ALP, and TBIL for non-HCC patients and AST and tumor size for HCC patients. Then, the AUCs of serum PIVKA-II for the diagnosis of early-stage HCC and late-stage HCC were further analyzed for each subgroup of patients by etiology, ALP, TBIL, and AST.

Table 2 Factors associated with abnormally elevated protein induced by vitamin K absence or antagonist-II by univariate and multivariate logistic analysis in non-hepatocellular carcinoma patients

Non-HCC	Univariate		Multivariate	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Age (yr)	1.009 (0.995, 1.024)	0.195		
Gender (M)	2.102 (1.308, 3.379)	0.002		
Etiology		< 0.001		< 0.001
NAFLD	-		-	
HBV	4.556 (1.089, 19.055)		3.214 (0.762, 13.548)	
HCV	1.687 (0.227, 12.571)		1.528 (0.204, 11.442)	
ALD	15.577 (3.542, 68.507)		9.883 (2.216, 44.086)	
AILD	4.295 (0.849, 21.729)		1.993 (0.368, 10.792)	
Cirrhosis (+)	1.848 (1.250, 2.731)	0.002		
ALT/ULN	1.026 (0.991, 1.063)	0.150		
AST/ULN	1.076 (1.029, 1.126)	0.001		
ALP/ULN	2.630 (1.823, 3.795)	< 0.001	2.146 (1.429, 3.221)	< 0.001
GGT/ULN	1.105 (1.050, 1.163)	< 0.001		
TBIL/ULN	1.228 (1.142, 1.321)	< 0.001	1.162 (1.080, 1.250)	< 0.001
ALB/LLN	0.063 (0.024, 0.166)	< 0.001		
PLT/LLN	0.618 (0.445, 0.857)	0.004		

HCC: Hepatocellular carcinoma; OR: Odds ratio; CI: Confidence interval; M: male; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NAFLD: Nonalcoholic fatty liver disease; ALD: Alcohol-related liver disease; AILD: Autoimmune liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyltranspeptidase; TBIL: Total bilirubin; ALB: Albumin; PLT: Platelet; ULN: Upper limit of normal; LLN: Lower limit of normal.

In Table 4, serum PIVKA-II had the best performance for diagnosing early-stage HCC in TBIL ≤ 1 × ULN subgroup, and the AUC of PIVKA-II in TBIL ≤ 1 × ULN subgroup was significantly higher than that in TBIL > 1 × ULN subgroup (0.817 *vs* 0.669, $P = 0.015$). When compared with the subgroup of nonviral liver disease, the AUC of PIVKA-II in the subgroup of viral liver disease for diagnosing early-stage HCC was only marginally higher (0.783 *vs* 0.736, $P = 0.678$), the difference was not significant, nor was the difference between ALP ≤ 1 × ULN and ALP > 1 × ULN (0.783 *vs* 0.729, $P = 0.398$). However, the AUC of PIVKA-II in AST ≤ 1 × ULN subgroup was almost the same to AST > 1 × ULN subgroup (0.774 *vs* 0.778, $P = 0.941$) in diagnosing early-stage HCC. Figure 3 shows the corresponding ROC curves of PIVKA-II for diagnosing early-stage HCC in different subgroups.

However, subsequent analysis of Supplementary Table 2 showed that the above factors, including etiology, ALP, TBIL, and AST, had almost no influence on the performance of PIVKA-II for the diagnosis of late-stage HCC. The AUCs of PIVKA-II did not differ significantly between subgroups in etiology (viral *vs* nonviral), ALP (≤ 1 × ULN *vs* > 1 × ULN), TBIL (≤ 1 × ULN *vs* > 1 × ULN) and AST (≤ 1 × ULN *vs* > 1 × ULN) (P all > 0.05). Supplementary Figure 2 also shows the corresponding ROC curves of PIVKA-II for diagnosing late-stage HCC in different subgroups.

Furthermore, we also analyzed the AUCs of PIVKA-II for HCC between viral and ALD etiologies. Supplementary Figure 3 shows that compared to the subgroup of viral etiology, the AUC of PIVKA-II for early-stage HCC was lower in ALD etiology (0.783 *vs* 0.655, $P = 0.470$), and also for late-stage HCC (0.970 *vs* 0.909, $P = 0.361$).

Serum PIVKA-II had better performance in diagnosing early-stage HCC for patients with TBIL ≤ 1 × ULN, both of viral etiology and HBV alone

Different etiologies of liver disease might induce diverse progression of liver injuries and changes in indicators of liver function, such as serum ALP and TBIL. Then, we further analyzed the AUCs of ROC curves of PIVKA-II by each subgroup of ALP and TBIL in patients of viral etiology and HBV alone, respectively, but not in patients with nonviral etiology on account of the limited cases of HCC.

Figure 4 shows that patients with TBIL ≤ 1 × ULN of viral etiology had substantially higher AUC of PIVKA-II for diagnosing early-stage HCC (0.837 *vs* 0.677, $P = 0.012$) than patients with TBIL > 1ULN.

Table 3 Factors associated with abnormally elevated protein induced by vitamin K absence or antagonist-II by univariate and multivariate logistic analyses in hepatocellular carcinoma patients

HCC	Univariate		Multivariate	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Age (yr)	1.002 (0.977, 1.027)	0.895		
Gender (M)	1.692 (0.726, 3.944)	0.223		
Etiology (viral)	1.008 (0.319, 3.185)	0.989		
Cirrhosis (+)	0.712 (0.280, 1.810)	0.475		
ALT/ULN	2.242 (1.235, 4.068)	0.008		
AST/ULN	2.745 (1.573, 4.788)	< 0.001	1.759 (1.072, 2.887)	0.025
ALP/ULN	3.542 (1.478, 8.490)	0.005		
GGT/ULN	1.436 (1.120, 1.843)	0.004		
TBIL/ULN	1.287 (0.947, 1.749)	0.108		
ALB/LLN	0.202 (0.042, 0.974)	0.046		
PLT/LLN	1.558 (0.985, 2.464)	0.058		
Tumor size (cm)	1.394 (1.221, 1.592)	< 0.001	1.349 (1.175, 1.549)	< 0.001
Number of tumors (1/2-3/> 3)	2.242 (1.340, 3.752)	0.002		
Vascular invasion (+)	5.907 (1.772, 19.696)	0.004		
Extrahepatic metastases (+)	3.551 (0.452, 27.887)	0.228		

HCC: Hepatocellular carcinoma; OR: Odds ratio; CI: Confidence interval; M: Male; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyltranspeptidase; TBIL: Total bilirubin; ALB: Albumin; PLT: Platelet; ULN: Upper limit of normal; LLN: Lower limit of normal.

Also, for patients with viral etiology, the AUC of PIVKA-II in ALP ≤ 1 × ULN was higher (0.800 *vs* 0.731, $P = 0.318$) than that in ALP > 1 × ULN, but no statistical significance was found. Supplementary Table 3 further shows the detailed value of AUC, best cutoff value, sensitivity, specificity, LR+, and LR- in each subgroup.

Furthermore, similar tendencies were also validated in patients with HBV alone. As shown in Supplementary Table 4, for patients with HBV alone, the AUCs of PIVKA-II were 0.832 and 0.676 ($P = 0.015$) in TBIL ≤ 1 × ULN and TBIL > 1 × ULN, and 0.794 and 0.732 ($P = 0.368$) in ALP ≤ 1 × ULN and ALP > 1 × ULN, respectively.

When the cutoff value of PIVKA-II for detecting early-stage HCC was set at 40 mAU/mL (ULN), the sensitivity, specificity, LR+, and LR- were further analyzed in patients with viral etiology and HBV alone. As shown in Table 5, compared to TBIL > 1 × ULN of viral etiology, the sensitivity of PIVKA-II in TBIL ≤ 1 × ULN of viral etiology increased from 30.30% to 57.47%, and the LR+ increased from 3.80 to 6.66, without compromising the corresponding specificity (92.02% *vs* 91.37%). Similarly, for patients with HBV alone, the sensitivity of PIVKA-II in TBIL ≤ 1 × ULN was also significantly higher than that in TBIL > 1 × ULN (57.14% *vs* 30.77%), as well as LR+ (6.16 *vs* 3.73), with similar specificity (90.72% *vs* 91.76%). Noticeably, the sensitivities in TBIL ≤ 1 × ULN have consistently been greater than those in ALP≤ 1 × ULN, both for patients with viral etiology (57.47% *vs* 52.88%) and HBV alone (57.14% *vs* 53.00%), and the corresponding specificities were almost the same. However, this tendency disappeared, and the sensitivities tended to be the same between TBIL > 1 × ULN and ALP > 1 × ULN, regardless of viral etiology (30.30% *vs* 30.61%) and HBV alone (30.77% *vs* 30.61%).

DISCUSSION

In the current study, 1051 patients in total were analyzed, and we determined the independent variables linked to elevated serum PIVKA-II levels in CLD and HCC patients, with the purpose of expounding their influence on the PIVKA-II performance for early-stage HCC and late-stage HCC detection. For patients with CLD, abnormally increased PIVKA-II levels were independently positively associated with ALD etiology, serum ALP and TBIL, but serum AST and tumor size for HCC patients. Compared to subgroups of nonviral etiology, ALP > 1 × ULN, TBIL > 1 × ULN and AST > 1 × ULN, serum PIVKA-II levels were significantly lower in subgroups of viral etiology, ALP ≤ 1 × ULN, TBIL ≤ 1 × ULN, and

Table 4 Performance characteristics of serum protein induced by vitamin K absence or antagonist-II in diagnosing early-stage hepatocellular carcinoma in different subgroups

Early HCC	AUC (95%CI)	Cutoff value (mAU/mL)	Se (%)	Sp (%)	LR+	LR-	*P* value
All early	0.765 (0.714, 0.815)	41.0	60.63	85.08	4.06	0.46	
Etiology							0.678
Viral	0.783 (0.732, 0.835)	42.8	58.97	87.73	4.81	0.47	
Nonviral	0.736 (0.519, 0.952)	177.0	60.00	94.18	10.31	0.42	
ALP							0.398
≤ 1 ULN	0.783 (0.725, 0.840)	41.0	58.42	89.95	5.81	0.46	
> 1 ULN	0.729 (0.618, 0.841)	42.0	69.23	71.07	2.39	0.43	
TBIL							0.015
≤ 1 ULN	0.817 (0.762, 0.872)	41.0	60.67	91.73	7.34	0.43	
> 1 ULN	0.669 (0.563, 0.775)	42.0	60.53	73.19	2.26	0.54	
AST							0.941
≤ 1 ULN	0.774 (0.712, 0.836)	41.0	57.95	88.08	4.86	0.48	
> 1 ULN	0.778 (0.691, 0.864)	41.0	66.67	82.41	3.79	0.40	

AUC: Area under the ROC curve; CI: Confidence interval; Se: Sensitivity; Sp: Specificity; LR+: Positive likelihood ratio; LR-: Negative likelihood ratio; ALP: Alkaline phosphatase; TBIL: Total bilirubin; AST: Aspartate aminotransferase; ULN: Upper limit of normal.

Table 5 Sensitivity, specificity, positive/negative likelihood ratio of protein induced by vitamin K absence or antagonist-II in diagnosing early-stage hepatocellular carcinoma at the cutoff value of 40 mAU/mL in patients with viral etiology and hepatitis B virus alone, respectively

	Cutoff value (mAU/mL)	Viral etiology				HBV			
		Se (%)	Sp (%)	+LR	-LR	Se (%)	Sp (%)	+LR	-LR
ALP									
≤ 1ULN	40	52.88	91.39	6.14	0.52	53.00	90.80	5.76	0.52
1ULN	40	30.61	92.45	4.06	0.75	30.61	92.23	3.94	0.75
TBIL									
≤ 1ULN	40	57.47	91.37	6.66	0.47	57.14	90.72	6.16	0.47
1ULN	40	30.30	92.02	3.80	0.76	30.77	91.76	3.73	0.75

HBV: Hepatitis B virus; Se: Sensitivity; Sp: Specificity; LR+: Positive likelihood ratio; LR-: Negative likelihood ratio; ALP: Alkaline phosphatase; TBIL: Total bilirubin; ULN: Upper limit of normal.

AST ≤ 1 × ULN in CLD patients; however, these differences disappeared in early-stage HCC. Furthermore, serum PIVKA-II in a subgroup of TBIL ≤ 1 × ULN always had the highest AUCs and the best performance in detecting early-stage HCC than TBIL > 1 × ULN, irrespective of all patients, viral etiology or HBV alone. However, the above factors, including etiology, ALP, TBIL, and AST, had almost no influence on PIVKA-II performance for late-stage HCC detection.

Four previous studies found that ALD patients had higher levels of PIVKA-II compared to viral hepatitis-related CLD patients[23-26], and ALD was also verified to be a significant factor related to positive serum PIVKA-II by a retrospective case-control study[26]. Consistently, in this study, patients with CLD with ALD etiology had the highest median level of PIVKA-II compared to those with other etiologies of HBV, HCV, NAFLD, and AILD, and was demonstrated to be independently associated with abnormally elevated PIVKA-II levels. A recent study enrolled 130 cases and showed that patients with CHB, CHC, and nonviral CLD had no significant differences in PIVKA-II levels (32 mAU/mL *vs* 35 mAU/mL *vs* 35 mAU/mL, any two $P > 0.05$)[31]. In the current study, however, a significant difference was observed in PIVKA-II levels between viral and nonviral CLD patients; meanwhile, PIVKA-II levels in NAFLD and ALD patients were significantly higher compared to those in HBV and HCV patients,

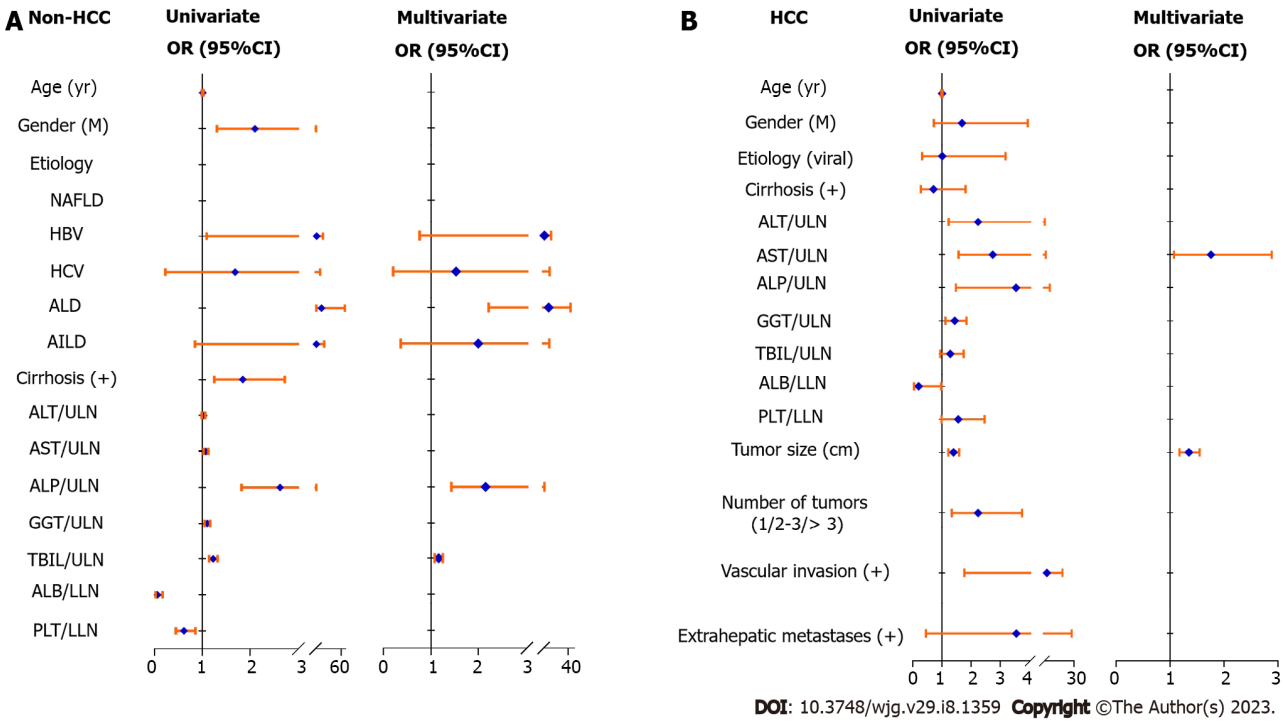


Figure 1 Forest plots of factors associated with abnormally elevated protein induced by vitamin K absence or antagonist-II by univariate and multivariate logistic analysis in patients with non-hepatocellular carcinoma and hepatocellular carcinoma. A: In non- hepatocellular carcinoma (HCC) patients; B: In HCC patients. HBV: Hepatitis B virus; HCV: Hepatitis C virus; NAFLD: Nonalcoholic fatty liver disease; ALD: Alcohol-related liver disease; AILD: Autoimmune liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; ULN: Upper limit of normal; TBIL: Total bilirubin; PLT: Platelet; LLN: Lower limit of normal; 95%CI: 95% confidence interval; OR: Odds ratio.

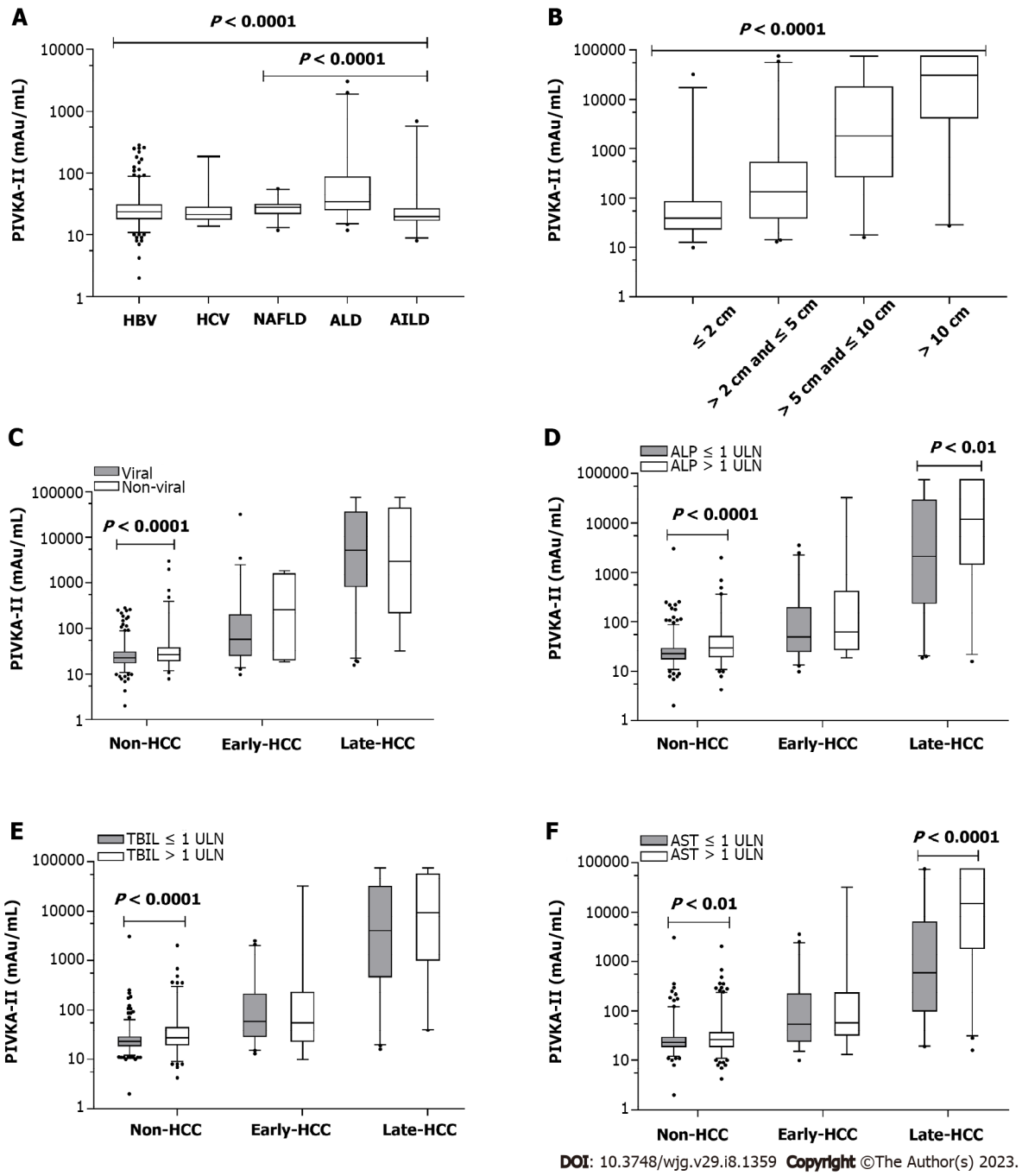
and AILD patients had the lowest PIVKA-II levels than those of other CLD patients.

In this study, tumor size was an independent factor relevant to abnormally elevated PIVKA-II levels in HCC patients, and the median levels of PIVKA-II gradually significantly increased as the tumor size changed by ≤ 2 cm, > 2 cm and ≤ 5 cm, > 5 cm and ≤ 10 cm to > 10 cm, which was consistent with the results of previous studies[32,33]. Moreover, the number of tumors and vascular invasion were associated with the abnormal elevation of PIVKA-II level which was only perceived in the univariate analysis. The results imply that tumor size had more influence on the PIVKA-II level than the number of tumors and vascular invasion.

Prior researches had reported that PIVKA-II serum levels are not elevated in hepatic flares and are influenced by liver regeneration triggered by necroinflammation in patients with active CLD, and have paid little attention to the confounding factors of liver injury and function that influence the levels of PIVKA-II[12,31]. Nonetheless, this is the first study to our knowledge to demonstrate that the abnormal PIVKA-II levels are significantly associated with serum ALP and TBIL in CLD patients and serum AST in HCC patients. Furthermore, in patients with CLD, the median PIVKA-II levels differed significantly between the subgroups of ALP ≤ 1 × ULN and ALP > 1 × ULN, as well as between TBIL ≤ 1 × ULN and TBIL > 1 × ULN; and in patients with late-stage HCC, the differences in PIVKA-II median levels were also observed between the subgroups of ALP and AST classified by 1 × ULN. However, these changes in PIVKA-II serum levels disappeared in early-stage HCC patients in the same situation.

Regretfully, the underlying mechanism behind the associations of ALD, ALP, and TBIL with elevated PIVKA-II levels in CLD patients remains unclear. Although vitamin K insufficiency may arise in chronic alcoholics[34], prior studies[23,25] found there was no direct correlation between PIVKA-II serum levels and vitamin K serum concentration. For ALP and TBIL, one likely explanation is that abnormalities in serum ALP and TBIL were always seen in all types of liver disorders and cholestasis. Vitamin K deficiency, as one of the fat-soluble vitamin deficiencies, is also a typical complication in chronic cholestasis patients, and vitamin levels were inversely correlated with serum TBIL levels[35-37]. Interestingly, one recent study also reported that hepatitis E patients in the raised PIVKA-II subgroup had significantly greater TBIL levels than those in the normal PIVKA-II subgroup ($P < 0.05$), and the trend of changes in serum PIVKA and TBIL were similar and related to the disease course[38].

It has been reported that the PIVKA-II's AUC for HCC in the CHB group was the highest compared to those in CHC and nonviral CLD patients (0.833 *vs* 0.732 *vs* 0.806)[31]. Similarly, in the current study, the AUC of PIVKA-II was slightly higher in patients with viral etiology compared to that in patients with nonviral etiology, irrespective of whether the HCC is in its early or late stages. Patients with ALD etiology had much lower AUCs of PIVKA-II for detecting HCC than patients with viral etiology,

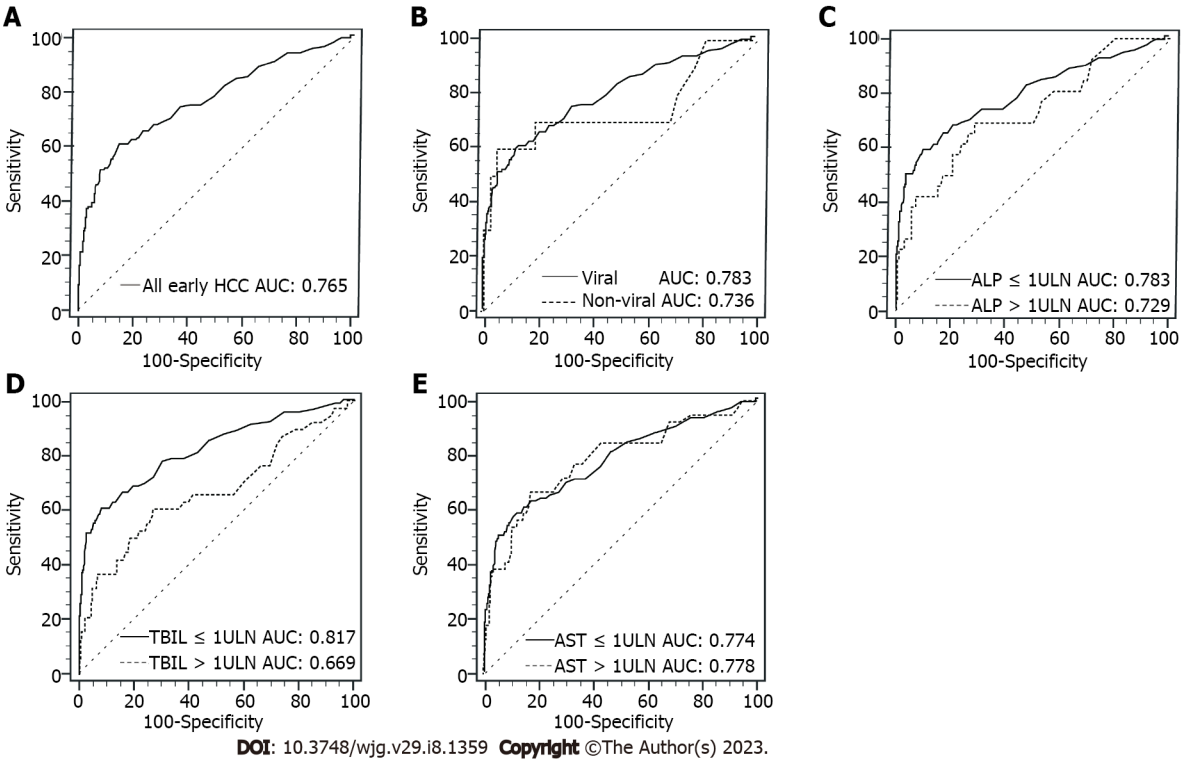


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Figure 2 Distributions and comparisons of serum protein induced by vitamin K absence or antagonist-II in different subgroups. A: Different etiologies of hepatitis B virus (HBV), HCV, nonalcoholic fatty liver disease, alcohol-related liver disease (ALD), autoimmune liver disease; B: Different tumor sizes of ≤ 2 cm (n = 60), > 2 cm and ≤ 5 cm (n = 88), > 5 cm and ≤ 10 cm (n = 65), > 10 cm (n = 54); C: Subgroups between viral and nonviral liver diseases; D: Subgroups between alkaline phosphatase (ALP) ≤ 1 × upper limit of normal (ULN) and ALP > 1 × ULN; E: Subgroups between total bilirubin (TBIL) ≤ 1 × ULN and TBIL > 1 × ULN; F: Subgroups between aspartate aminotransferase (AST) ≤ 1 × ULN and AST > 1 × ULN. HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NAFLD: Nonalcoholic fatty liver disease; ALD: Alcohol-related liver disease; AILD: Autoimmune liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; ULN: Upper limit of normal; TBIL: Total bilirubin.

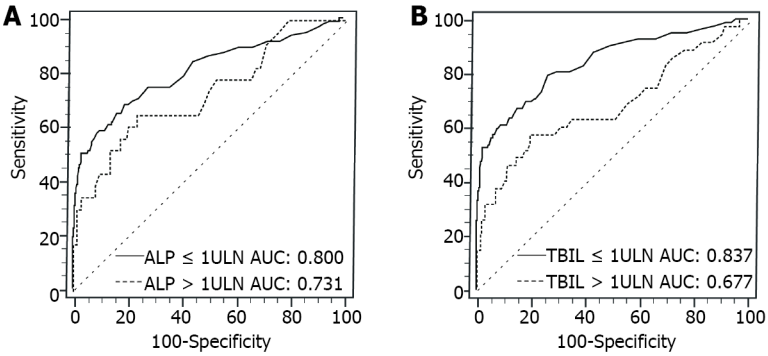
especially for early-stage HCC. However, in previous literature, the influences of serum ALP and TBIL on the performance of PIVKA-II for the detection of HCC have not been evaluated. In this study, we provided evidence that patients in TBIL ≤ 1 × ULN subgroup had the best performance of PIVKA-II for detecting early-stage HCC, and the AUC of PIVKA-II in TBIL ≤ 1 × ULN subgroup was significantly higher compared to that in TBIL > 1 × ULN subgroup. Between ALP ≤ 1 × ULN and ALP > 1 × ULN, a similar trend was also observed, although the difference was not significant. However, no significant influences of serum ALP and TBIL were observed on the performance of PIVKA-II for late-stage HCC.

Further analysis also showed that the above changes and differences in the performance of PIVKA-II for the detection of early-stage HCC still existed between different subgroups of ALP and TBIL in patients with viral etiology and HBV alone. Moreover, when 40 mAU/mL (ULN) was set as the cutoff



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Figure 3 Comparisons of receiver operating characteristic curves of serum protein induced by vitamin K absence or antagonist-II for diagnosing early-stage hepatocellular carcinoma in different subgroups. A: For all early-stage patients; B: Between viral and nonviral subgroups; C: Between alkaline phosphatase ≤ 1 × upper limit of normal (ULN) and alkaline phosphatase > 1 × ULN subgroups; D: Between total bilirubin (TBIL) ≤ 1 × ULN and TBIL > 1 × ULN subgroups; E: Between aspartate aminotransferase (AST) ≤ 1 × ULN and AST > 1 × ULN subgroups. ULN: Upper limit of normal; TBIL: Total bilirubin; HCC: Hepatocellular carcinoma; AUC: Area under the receiver operating characteristic curve; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase.



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Figure 4 Comparisons of receiver operating characteristic curves of serum protein induced by vitamin K absence or antagonist-II for diagnosing early-stage hepatocellular carcinoma in subgroups of alkaline phosphatase and total bilirubin for patients with viral etiology. A: Between alkaline phosphatase (ALP) ≤ 1 × upper limit of normal (ULN) and ALP > 1 × ULN subgroups; B: Between total bilirubin (TBIL) ≤ 1 × ULN and TBIL > 1 × ULN subgroups. ULN: Upper limit of normal; TBIL: Total bilirubin; ALP: Alkaline phosphatase; AUC: Area under the receiver operating characteristic curve.

value of PIVKA-II, for patients with TBIL ≤ 1 × ULN, serum PIVKA-II had the highest sensitivities in detecting early-stage HCC than other subgroups and enough high specificities at the same time, irrespective of viral etiology or HBV alone. These results strongly suggest that serum PIVKA-II would have better performance in detecting HCC at an early-stage for patients with normal serum TBIL.

The current study had a number of limitations. Firstly, it was a retrospective design, and some unidentified potential biases might exist, although we completely ruled out the possible interferences on serum PIVKA-II by the potential confounding factors in the exclusion criteria. Then, the limited sample size, including HCC patients with CHC, ALD, NAFLD, and AILD, does not allow us to independently evaluate the influences of these factors on the performance of PIVKA-II in HCC patients with CHC, NAFLD, and AILD, and decrease the reliability of that in HCC patients with ALD. Finally, further

multicenter research with more participants are required to validate the above findings.

CONCLUSION

The present study suggests that abnormally elevated PIVKA-II levels were independently positively associated with ALD etiology, serum ALP and TBIL for non-HCC CLD patients and serum AST and tumor size for HCC patients. Better performance of PIVKA-II for discriminating HCC at an early stage from patients with CLD would be achieved in patients with normal TBIL, and more attention should be given to the availability of PIVKA-II in HCC surveillance for at-risk patients with elevated serum TBIL.

ARTICLE HIGHLIGHTS

Research background

Serum protein induced by vitamin K absence or antagonist-II (PIVKA-II) is a promising biomarker for hepatocellular carcinoma (HCC) surveillance.

Research motivation

Investigate those unclear factors regarding different liver disease etiologies, the liver injury severity, and disease activity associated with the abnormal levels of serum PIVKA-II in chronic liver disease (CLD) and HCC patients.

Research objectives

Identify the potential contributing factors and evaluate their influence on serum PIVKA-II levels and the performance of PIVKA-II for the diagnosis of HCC in different populations.

Research methods

This study retrospectively enrolled in 784 CLD patients and 267 HCC patients in Mengchao Hepatobiliary Hospital of Fujian Medical University from April 2016 to December 2019. Logistic regression and the area under the receiver operating characteristic curve (AUC) were used to evaluate the influencing factors and diagnostic performance of PIVKA-II for HCC, respectively.

Research results

Elevated PIVKA-II levels were independently positively associated with alcohol-related liver disease (ALD), serum alkaline phosphatase (ALP), and total bilirubin (TBIL) for CLD patients and aspartate aminotransferase (AST) and tumor size for HCC patients (all $P < 0.05$). Serum PIVKA-II were significantly lower in patients with viral etiology, ALP ≤ 1 × upper limit of normal (ULN), TBIL ≤ 1 × ULN, and AST ≤ 1 × ULN than in those with nonviral disease and abnormal ALP, TBIL, or AST (all $P < 0.05$), but the differences disappeared in patients with early-stage HCC. For patients with TBIL ≤ 1 × ULN, the AUC of PIVKA-II was significantly higher compared to that in patients with TBIL > 1 × ULN (0.817 *vs* 0.669, $P = 0.015$), while the difference between ALP ≤ 1 × ULN and ALP > 1 × ULN was not statistically significant found between ALP ≤ 1 × ULN and ALP > 1 × ULN (0.783 *vs* 0.729, $P = 0.398$). These trends were then more prominently perceived in subgroups of patients with viral etiology and HBV alone.

Research conclusions

Abnormally elevated PIVKA-II levels were independently positively associated with ALD etiology, serum ALP and TBIL for non-HCC CLD patients and serum AST and tumor size for HCC patients. Better performance of PIVKA-II for discriminating HCC at an early stage from patients with CLD would be achieved in patients with normal TBIL.

Research perspectives

More attention should be given to the availability of PIVKA-II in HCC surveillance for at-risk patients with elevated serum TBIL, which may be important for surveillance counseling of early-stage HCC.

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FOOTNOTES

Author contributions: Qian XJ, Wen ZM, and Huang XM contributed to acquisition of data, analysis and interpretation of data, drafting of the manuscript, and statistical analysis; Feng HJ, Li SC, Zhang Y, Peng WG, Yang JR, Zheng ZY, and Zhang L contributed to acquisition of data and critical revision of the manuscript for important intellectual content; Lin SS, Liu YN, and Zhang DW contributed to administrative, technical, material support, and critical revision of the manuscript for important intellectual content; Lu FM, Liu LJ, and Pan WD contributed to study concept and design, obtained funding and material support, study supervision and critical revision of the manuscript for important intellectual content; all authors of this research have approved the final version of the article; Qian XJ, Wen ZM, and Huang XM contributed equally to this work; Lu FM, Liu LJ, and Pan WD contributed equally to this work.

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Country/Territory of origin: China

ORCID number: Xiang-Jun Qian 0000-0001-6485-0731; Feng-Min Lu 0000-0002-1832-3209; Wei-Dong Pan 0000-0001-9519-7683.

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SYSTEMATIC REVIEWS

Burden of bone disease in chronic pancreatitis: A systematic review and meta-analysis

Ankit Chhoda, Maria Jose Hernandez-Woodbine, Nana Afua Akkya Addo, Syed Alishan Nasir, Alyssa Grimshaw, Craig Gunderson, Awais Ahmed, Steven D. Freedman, Sunil G. Sheth

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Ankit Chhoda, Maria Jose Hernandez-Woodbine, Awais Ahmed, Steven D. Freedman, Sunil G. Sheth, Department of Gastroenterology, Beth Israel Deaconess Medical Center, Boston, MA 02215, United States

Nana Afua Akkya Addo, Syed Alishan Nasir, Department of Medicine, Norwalk Hospital, Yale School of Medicine, Norwalk, CT 06850, United States

Alyssa Grimshaw, Cushing/Whitney Medical Library, Yale University, New Haven, CT 06510, United States

Craig Gunderson, General Internal Medicine, Yale School of Medicine, New Haven, CT 06510, United States

Corresponding author: Sunil G. Sheth, AGAF, FACG, FASGE, MBBS, MD, Associate Professor, Department of Gastroenterology, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02215, United States. ssheth@bidmc.havard.edu

Abstract

BACKGROUND

Bone disease is an under-recognized cause of morbidity in chronic pancreatitis (CP). Over the past decade, publications of original studies on bone disease in CP has warranted synthesis of the evidence to ascertain the true burden of the problem.

AIM

To quantify the prevalence of osteopenia, osteoporosis, and fragility fractures in CP patients and investigate the associated clinical features and outcomes.

METHODS

A systematic search identified studies investigating bone disease in CP patients from Cochrane Library, Embase, Google Scholar, Ovid Medline, PubMed, Scopus, and Web of Science, from inception until October 2022. The outcomes included prevalence of osteopenia, osteoporosis, and fragility fractures, which were meta-analyzed using a random-effects model and underwent metaregression to delineate association with baseline clinical features.

RESULTS

Twenty-one studies were included for systematic review and 18 studies were

included for meta-analysis. The pooled prevalence of osteopenia and osteoporosis in CP patients was 41.2% (95%CI: 35.2%-47.3%) and 20.9% (95%CI: 14.9%-27.6%), respectively. The pooled prevalence of fragility fractures described among CP was 5.9% (95%CI: 3.9%-8.4%). Meta-regression revealed significant association of pancreatic enzyme replacement therapy (PERT) use with prevalence of osteoporosis [coefficient: 1.7 (95%CI: 0.6-2.8); $P < 0.0001$]. We observed no associations with mean age, sex distribution, body mass index, alcohol or smoking exposure, diabetes with prevalence of osteopenia, osteoporosis or fragility fractures. Paucity of data on systemic inflammation, CP severity, and bone mineralization parameters precluded a formal meta-analysis.

CONCLUSION

This meta-analysis confirms significant bone disease in patients with CP. Other than PERT use, we observed no patient or study-specific factor to be significantly associated with CP-related bone disease. Further studies are needed to identify confounders, at-risk population, and to understand the mechanisms of CP-related bone disease and the implications of treatment response.

Key Words: Chronic pancreatitis; Fractures; Osteoporosis; Osteopenia; Bone disease

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Core Tip: Bone disease is an under recognized cause of morbidity in chronic pancreatitis (CP). This systematic review and meta-analysis demonstrate substantial burden of bone disease (osteopenia and osteoporosis) and fragility fractures in CP patients. In addition, metaregression has demonstrated a significant association of osteoporosis with pancreatic enzyme replacement. The study is powered by high-quality studies with large sample size and clearly defined study population and outcome measures.

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INTRODUCTION

Chronic pancreatitis (CP) is a progressive, multifactorial fibro-inflammatory syndrome, which arises from persistent pathological response to noxious stimuli[1]. It is characterized by chronic abdominal pain, exocrine and endocrine insufficiency. Over time, nutritional deficiencies, systemic inflammation, and etiological factors like alcohol or smoking may disrupt the balance between bone formation and resorption[2]. These maladaptive alterations may subsequently cause bone disease *i.e.*, osteopenia and osteoporosis, leading to fractures which result from low energy trauma, called fragility fractures, and have significant implications on quality of life[3].

Osteoporosis is a public health hazard with a substantial economic burden[4]. Recent data from the National Health and Nutrition Examination Survey found its age-adjusted prevalence among adults older than 50 years to be 12.6% affecting more women than men (19.6% *vs* 4.4%). In view of the burden of bone disease and subsequent fracture risk in individuals affected by CP, various consensus guidelines have recommended baseline bone health assessment[5,6]. The adherence to guidelines has been low and studies have demonstrated that less than a quarter of CP patients are screened for bone disease, suggesting that bone disease is an underappreciated source of morbidity in CP[7,8].

A previous systematic review by Duggan *et al*[9] estimated the prevalence of osteopenia and osteoporosis in patients with CP to be 39.8% and 23.4%, respectively. The analysis was based on a limited number of studies ($n = 11$) and did not quantify fragility fractures which are complications of CP-related bone disease. Over the last decade, multiple large-sized studies including those from multicenter cohorts have investigated bone disease in CP as well as the nutritional, anthropometric, and inflammatory parameters which may impact bone health outcomes. Thus, with quantitative data on covariates and fragility fractures available, an updated synthesis of evidence is pertinent. This systematic review and meta-analysis aims to quantify the prevalence of osteopenia, osteoporosis, and fragility fractures in CP patients and to delineate clinical parameters which impact their occurrence.

MATERIALS AND METHODS

Protocol registration

This systematic review was performed in accordance with PRISMA guidelines[10]. The review methodology was pre-registered at open-source forum[11].

Search strategy

A systematic search of the literature was conducted by a medical librarian in the following databases: Cochrane Library, Ovid Embase, Google Scholar, Ovid Medline, PubMed, Scopus, and Web of Science Core Collection to find articles published from the inception of the database to October 20, 2022. The search was performed using a combination of controlled and free text terms for CP and bone diseases (strategy outlined in Supplementary Table 1). The search was not limited to publication type or date. the search was peer-reviewed by a second librarian using peer review for electronic search strategies[12]. Citations were imported into an Endnote 20 Library (Version 20.2, Clarivate Analytics, Philadelphia), and after removal of duplicates, the remaining studies were imported into Covidence (Melbourne, Australia) for screening and data extraction.

Study selection

Two co-authors (MJ, AC) independently used the inclusion and exclusion criteria to assess titles and abstracts. Eligibility decisions and disagreements were reconciled through discussion with the senior author (SS). Full-text articles included by two reviewers underwent data extraction and quality assessment.

Inclusion criteria: This systematic review included original cross-sectional and cohort studies, which were of prospective and retrospective design. CP patients were diagnosed based on specific codes related to CP per the International Classification of Diseases (ICD-9/10) and through predefined clinical, radiologic and/or histologic findings (described in Table 1).

Exclusion criteria: The studies which did not report bone diseases in CP patients were ineligible for the systematic review. We also excluded studies published in foreign languages, associated with animal research, reviews, abstracts, letters, case reports, and series with a sample size of less than 10 patients were excluded (Figure 1).

Data extraction

We noted data on study design, population characteristics such as age, gender, method of CP diagnosis (ICD-code based *vs* systematic clinical-radiologic features), body mass index (BMI), and exposure to alcohol and smoking. These data were independently extracted by two authors (MJ and AC). The outcome of interest included the prevalence of osteopenia, osteoporosis, and fragility fractures among the CP patients. The definitions of these bone outcome measures were also noted and have been outlined in Table 2.

Meta-analysis methodology

Prevalence rates were pooled using random-effects meta-analysis and calculated using the score method [13]. Between-study heterogeneity was estimated using I^2 statistics wherein 25%, 50%, and 75% were considered to indicate low, moderate, and high heterogeneity, respectively. Sensitivity analysis was conducted by sequentially removing individual studies. Statistical analysis was performed using Stata/IC, version 17 (StataCorp, College Station, TX). Metaregression, subgroup analysis and publication bias assessment could not be performed for analyses including < 10 studies[14].

Metaregression

Metaregression using the restricted maximum likelihood method was also performed using variables of mean age, sex, BMI, smoking or alcohol exposure, diabetes, serum parathyroid hormone (PTH), percentage population with vitamin D deficiency, and pancreatic enzyme replacement therapy (PERT).

Study quality and publication bias assessment

The quality of observational studies was independently assessed using the Newcastle-Ottawa Scale by two investigators (SN and AA) and recorded in a Microsoft Excel spreadsheet (XP Professional Edition; Microsoft Corp, Redmond, WA, United States)[15]. Any discrepancy was resolved by the senior author (SS). Publication bias were assessed by inspection of a funnel plot and Egger's test[16].

Table 1 Description of study design and study population

Ref.	Design	CP patients	CP severity, *n* (%)	CP etiology, *n* (%)	Study population	Race	Female patients, *n* (%)	Age, mean ± SD	BMI, mean ± SD
Morán *et al*[17], 1997	Cross sectional	Clinicoradiological	All severe	Alcohol: 10 (71.4)	CP: 14		CP: 0	CP: 56[1] (-)	CP: 22.64
				Idiopathic 4 (28.6)	Controls: -		Controls: -	Controls: -	Controls: -
Haaber *et al*[18], 2000	Cross sectional	Clinicoradiological		Alcohol: 46 (79)	CP: 58	CP: -	CP: 26 (44.8)	CP: 53 (9)	CP: 23 (5)
					Controls: -	Controls: -	Controls: -	Controls: -	Controls: -
Dujsikova *et al* [19], 2008	Cross sectional	EUS based criteria	Wiersema classification: Mild: 41 (56.2), moderate: 12 (16.4), severe: 20 (27.4)		CP: 73	CP: -	CP:17 (23.28)	CP: 46.61 (13.23)	CP: -
					Controls: -	Controls: -	Controls: -	Controls: -	Controls: -
Tignor *et al*[20], 2010	Retrospective cohort	ICD-9 code 577.1			CP: 3192	CP: White: 2091 (65.5), black: 419 (13.1), hispanic: 222 (6.9), others: 532 (16.7)	CP: 1636 (51.25)	CP: -	CP: -
					Controls: 1436699	Control: White: 860190 (59.9), black: 115199 (8.0), hispanic: 102000, other: 451110	Controls: 907328 (63.15)	Controls: -	Controls: -
Sudeep *et al*[22], 2011	Cross sectional	Not defined		Tropical pancreatitis: 20 (65)	CP: 31	CP: -	CP: 0	CP: 35.8 (9)	CP: 18.46 (2.86)
				Idiopathic: 11 (35)	Controls: 35	Controls: -	Controls: 0	Controls: 38.6 (5.2)	Controls: 22.6 (3.1)
Joshi *et al*[21], 2011	Cross sectional	Clinicoradiological		All patients with tropical calcific pancreatitis	CP: 72	CP: -	CP: 34 (47.2)	CP: 31.1 (10.3)	CP: 19 (3.1)
					Controls: 100	Controls: -	Controls: 50 (50)	Controls: 32.6 (9.6)	Controls: 23.6 (3.2)
Duggan *et al* [23], 2012	Cross sectional	Clinicoradiological	Cambridge classification: Mild (37.1), severe (27.4)	Alcohol: 24 (38.7)	CP: 62	CP: -	CP: 17 (27.41)	CP: 47.9 (12.5)	CP: 25.6 (5)
				Idiopathic: 38 (61.3)	Controls: 66	Controls: -	Controls: 18 (27.27)	Controls: 47.74 (11)	Controls: 28.0 (4.1)
Sikkens *et al*[25], 2013	Prospective cohort	Clinicoradiological		Alcohol: 20 (50)	CP: 40	CP: -	CP: 17 (42.5)	CP: 52 (11)	CP: 24 (5)
				Idiopathic: 17 (43)	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -
				Other: 3 (7)					
Prabhakaran *et al* [28], 2014	Cross sectional	Clinicoradiological.	Cambridge classification: Mild (13.1), moderate (5.05), marked: (81.8)	Alcohol: 72 (70)	CP: 103	CP: -	CP: 0	CP: 38.6 (20.64)	CP: 19.7

				Idiopathic: 31 (29.1)	Controls: -	Controls: -	Controls: 0	Controls: 36.7 (20.70)	Controls: -
Bang *et al*[26], 2014	Prospective cohort	ICD-10: K86.0 (alcohol induced CP), K86.1 (other CP)			CP: 11972	CP: -	CP: 4011 (33.5)	CP: 54.5 (14)	CP: -
					Controls: 119720	Controls: -	Controls: 40106 (33.49)	Controls: 54.5 (14)	Controls: -
Duggan *et al* [27], 2015	Cross sectional	Clinicoradiological	Cambridge classification (unspecified number in each category)	Alcohol: 18 (62.1)	CP: 29	CP: -	CP: 12 (41.37)	CP: 44.3 (12.3)	CP: 25.2 (5.1)
				Idiopathic: 8 (27.6)	Controls: 29	Controls: -	Controls: 12 (41.37)	Controls: 45.8 (9.8)	Controls: 27.3 (3.7)
				Other: 3 (10.3)					
Munigala *et al* [24], 2016	Cross sectional	ICD-9 code 577.1			CP: 3257	CP: White 2120 (65), black 1012 (31), others 125 (4)	CP: 178 (5.46)	CP: 54.2 (11.1)	CP: -
					Controls: 450655	Controls: White: 325132 (72), black: 76031 (17), others: 49492 (11)	Controls: 53108 (11.78)	Controls: 53.6 (13.9)	Controls: -
Kumar *et al*[29], 2017	Cross sectional	Clinicoradiological			CP: 102	CP: -	CP: 17 (16.7)	CP: 40.8 (12.6)	CP: 22.5 (3.2)
					Controls: -	Controls: -	Controls: -	Controls: -	Controls: -
Stigliano *et al* [32], 2018	Cross sectional	M-ANNHEIM criteria	M-ANNHEIM scoring system: Minor: 74 (35), Increased: 99 (47), advanced: 32 (15), marked: 6 (3)	Alcoholic: 91 (43)	CP: 211	CP: -	CP: 69 (32.7)	CP: 60 (-)	CP: 24 (4)
				Idiopathic: 40 (19)	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -
				Hereditary: 8 (4)					
				Obstructive: 12 (5.7)					
Kuhlmann *et al* [30], 2018	Cross sectional	Score ≥ 4 points based on Lüneburg criteria			CP: 67	CP: -	CP: 27 (40.29)	CP: 60[1] (-)	CP: 22.7 (15-37.9)
					Controls: -	Controls: -	Controls: -	Controls: -	Controls: -
Min *et al*[31], 2018	Prospective cohort	EUS criteria and/or	EUS criteria (unspecified number in each category)	Toxic/metabolic: 54 (59.3)	CP: 91	CP: -	CP: 34 (37.36)	CP: 48.6 (10.4)	CP: 26.1 (7.8)
		secretin stimulation testing		Idiopathic: 17 (18.6)	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -
				Hereditary: 13 (14.3)					
				Autoimmune: 5 (5.5)					
Gupta *et al*[33], 2019	Prospective cohort	Clinicoradiological and EUS			CP: 38	CP: White 35 (92), black 3 (8)	CP: 19 (50)	CP: 44 (10.7)	CP: 26.7 (5.9)
					Controls: -	Controls: -	Controls: -	Controls: -	Controls: -

Kanakis *et al*[7], 2020	Retrospective cohort	Clinicoradiological			CP: 239 Controls: -	CP: White 43 (88), minorities: 6 (12) Controls: -	CP: 37 (15.48) Controls: -	CP: 56[1] (-) Controls: -	CP: 23 (8) Controls: -
Hart *et al*[34], 2021	Cross sectional	Clinicoradiological	Cambridge classification (unspecified number in each category)	-	CP: 282 Controls: -	CP: White race (87.2), minorities (12.8) Controls: -	CP: 145 (51.41) Controls: -	CP: 56[1] (-) Controls: -	CP: - Controls: -
Vujasinovic *et al* [8], 2021	Retrospective cohort	2002 Asia-Pacifc consensus report		Alcohol and smoking: 40 (33.9) Smoking only: 12 (11) Alcohol only: 7 (5.9) Hereditary: 21 (11.8) Immunological: 23 (14.4) Efferent duct factors: 11 (9.3)	CP: 118 Controls: -	CP: - Controls: -	CP: 49 (41.52) Controls: -	CP: 53.1 (16.3) Controls: -	CP: 23.9 (4.4) Controls: -
Tang *et al*[35], 2021	Cross sectional	ICD-9 based codes	M-ANNHEIM clinical stage 0: 6 (5.8), I: 59 (56.7), II: 26 (25.0), III: 8 (7.7), IV: 5 (4.8)		CP: 104 Controls: -	CP: - Controls: -	CP: 31 (29.8) Controls: -	CP: 46.08 (14.43) Controls: -	CP: 21.43 (2.85) Controls: -

[1]Number of subjects who consented to Dual-energy X-ray Absorptiometry scan if detailed in the studies

CP: Chronic pancreatitis; BMI: Body mass index; EUS: Endoscopic ultrasound; ICD: International Classification of Diseases.

RESULTS

Literature search

The literature search yielded 2081 results and after the removal of 20 duplicates, 2061 citations underwent title and abstract screening. The exclusion of 1979 citations due to lack of relevance to the research question resulted in 82 citations for full-text review. The review of their full texts enabled exclusion of 61 citations and finally 21 studies were included[7,8,17-35] (Supplementary Table 2). The excluded studies had incorrect study design (n = 27), wrong outcomes (n = 17), ineligible patient population (n = 8), non-English language (n = 6), wrong setting (n = 3), and duplicate center or overlapping populations (n = 1) (Supplementary Table 3). The search and selection processes are summarized in Figure 1.

Study design

All the eligible studies were observational studies and included 14 cross-sectional and 7 cohort studies (described in Table 1). Among the cohort studies, four were performed prospectively[25,26,31,33], and remaining three had retrospective design[7,8,35]. Most of the studies originated from European

Table 2 Characteristics of study outcomes, *n* (%)

Ref.	Population	Outcome definition	Osteoporosis	Osteopenia	Pathologic fracture
Morán *et al*[17], 1997	CP: 14	T score < -2.5	CP: 3 (21.4)	CP: 10 (71.4)	CP: -
	Controls: -	T score -1 to -2.5	Controls: -	Controls: -	Controls: -
		Unavailable			
Haaber *et al*[18], 2000	CP: 58	Z score < -2	CP: 13 (22.4)	CP: 36 (62)	CP: -
	Controls: -	Z score < -1.0	Controls: -	Controls: -	Controls: -
		Unavailable			
Dujsikova *et al*[19], 2008	CP: 73	T score < -2.5	CP: 4 (5.5)	CP: 19 (26)	CP: 1 (1.3)
	Controls: -	T score -1 to -2.5	Controls: -	Controls: -	Controls: -
		Undefined			
Tignor *et al*[20], 2010	CP: 3192	Not studied	CP: -	CP: -	CP: 154 (4.8)
	Controls: 1436699	Not studied	Controls: -	Controls: -	Controls: -
		Vertebral, hip, and wrist fractures using ICD-9 codes			
Sudeep *et al*[22], 2011	CP: 31	T score < -2.5	CP: 9 (29)	CP: -	CP: -
	Controls: 35	T score -1 to -2.5	Controls: 3 (8.5)	Controls: -	Controls: -
		Unavailable			
Joshi *et al*[21], 2011	CP: 72	Z score < -2	CP: 22 (30.5)	CP: -	CP: 0
	Controls: 100	Unavailable	Controls: -	Controls: -	Controls: -
		Undefined			
Duggan *et al*[23], 2012	CP: 62	T score < -2.5	CP: 18 (33)	CP: 21 (39.6)	CP: -
	Controls: 66	T score -1 to -2.5	Controls: 6 (10.1)	Controls: 20 (33.8)	Controls: -
		Unavailable			
Sikkens *et al*[25], 2013	CP: 40	T score < -2.5	CP: 4 (10)	CP: 18 (45)	CP: -
	Controls: -	T score -1 to -2.5	Controls: -	Controls: -	Controls: -
		Unavailable			
Prabhakaran *et al*[28], 2014	CP: 103	T score < -2.5	CP: 25 (30.1)	CP: 38 (45.7)	CP: -
	Controls: -	T score -1 to -2.5	Controls: -	Controls: -	Controls: -
		Unavailable			
Bang *et al*[26], 2014	CP: 11972	M80.0-M81.9 based on ICD-10 code	CP: 898 (7.5)	CP: -	CP: 1055 (8.8)
	Controls: 119720	Unavailable	Controls: 4070 (3.3)	Controls: -	Controls: 8485 (7)
		Spine, humerus, distal forearm, and proximal femur based on ICD-10 codes			
Duggan *et al*[27], 2015	CP: 29	T score < -2.5	CP: 9 (31)	CP: 13 (44.8)	CP: -
	Controls: 29	T score -1 to -2.5	Controls: 2 (6.8)	Controls: 15 (51.7)	Controls: -
		Unavailable			
Munigala *et al*[24], 2016	CP: 3257	Unspecified ICD-9 codes	CP: -	CP: -	CP: 153 (4.6)
	Controls: 450655	Unavailable	Controls: -	Controls: -	Controls: 9325 (2)
		ICD-9 codes: vertebral (805.2, 805.3, 805.4,805.5, 805.6, 805.7), hip (820.0, 820.1, 820.2, 820.3, 820.8, 820.9), or wrist fractures (814.0, 814.1, 813.4, 813.5)			

Kumar *et al*[29], 2017	CP: 102	Z score < -2	CP: 6 (5.8)	CP: 21 (20.5)	CP: -
	Controls: -	Unavailable	Controls: -	Controls: -	Controls: -
		Unavailable			
Stigliano *et al*[32], 2018	CP: 211	T score < -2.5	CP: 46 (21.8)	CP: 89 (42.1)	CP: 13 (6.1)
	Controls: -	T score: -1 to -2.5	Controls: -	Controls: -	Controls: -
		Occurring at the spine, hip and distal radius, and not associated with traumatic events			
Kuhlmann *et al* [30], 2018	CP: 67	T score < -2.5	CP: 18 (26.8)	CP: 34 (50.7)	CP: -
	Controls: -	T score: -1 to -2.5	Controls: -	Controls: -	Controls: -
		Unavailable			
Min *et al*[31], 2018	CP: 91	T score < -2.5	CP: 10 (22.2)	CP: 21 (46.6)	CP: -
	Controls: -	T score: -1 to -2.5	Controls: -	Controls: -	Controls: -
		Unavailable			
Gupta *et al*[33], 2019	CP: 38	T score < -2.5	CP: 21 (55.2)	CP: -	CP: -
	Controls: -	T score: -1 to -2.5	Controls: -	Controls: -	Controls: -
		A fall from standing height or less that resulted in a fracture			
Kanakis *et al*[7], 2020	CP: 239	T score < -2.5 or history of fragility fracture	CP: 15 (30.6)	CP: 27 (55.1)	CP: 22 (9)
	Controls: -	T score: -1 to -2.5	Controls: -	Controls: -	Controls: -
		Hip or vertebral fracture not due to excess trauma			
Hart *et al*[34], 2021	CP: 282	T score < -2.5	CP: 48 (17)	CP: 110 (39)	CP: 6 (2.1)
	Controls: -	T score: -1 to -2.5	Controls: -	Controls: -	Controls: -
		Spontaneous fractures			
Vujasinovic *et al* [8], 2021	CP: 118	T score < -2.5	CP: 30 (25.4)	CP: 33 (27.9)	CP: 33 (27.9)
	Controls: -	T score: -1 to -2.5	Controls: -	Controls: -	Controls: -
		Unavailable			
Tang *et al*[35], 2021	CP: 104	T score < -2.5	CP: 6 (5.7)	CP: 32 (30.7)	CP: -
	Controls: -	T score: -1 to -2.5	Controls: -	Controls: -	Controls: -
		Occurring in the hip, spine, or wrist			

CP: Chronic pancreatitis; ICD: International Classification of Diseases.

countries (n = 9), while the remaining were from the United States (n = 6), India (n = 4), China (n = 1), and Argentina (n = 1). Multicenter collaboration was noted in two eligible studies whereas the remaining 19 were performed at single centers. The controls of matched patients were also analyzed in seven studies which were from a healthcare system (n = 3)[20,22,24]; healthy community population (n = 3)[21,26,27]; or were not defined (n = 1)[28].

Population characteristics

The qualitative review of 21 studies included 20155 CP patients and 2007278 control patients (from seven controlled studies). The patients with CP were identified based on the combination of clinical, radiologic, endoscopic and/or histologic findings in 17 studies[7,8,17-19,21-23,25,27-34], and diagnosed through ICD-9/10 codes in four studies[20,24,26,35] (Table 1).

Prevalence of bone disease

We performed meta-analysis of 21 eligible studies which included 20155 CP patients evaluated for bone disease.

Prevalence of osteopenia in CP patients: In adult CP patients, the pooled prevalence of osteopenia was 41.2% (95%CI: 35.2%-47.3%, P < 0.0001; χ^2 = 63.0, df = 14, P < 0.0001; I^2 = 77.8%, τ^2 = 0.04)[7,8,17-19,23,25,27-32,34,35] (Figure 2A). All of the studies prevalence estimates had high heterogeneity since I^2 > 75%.

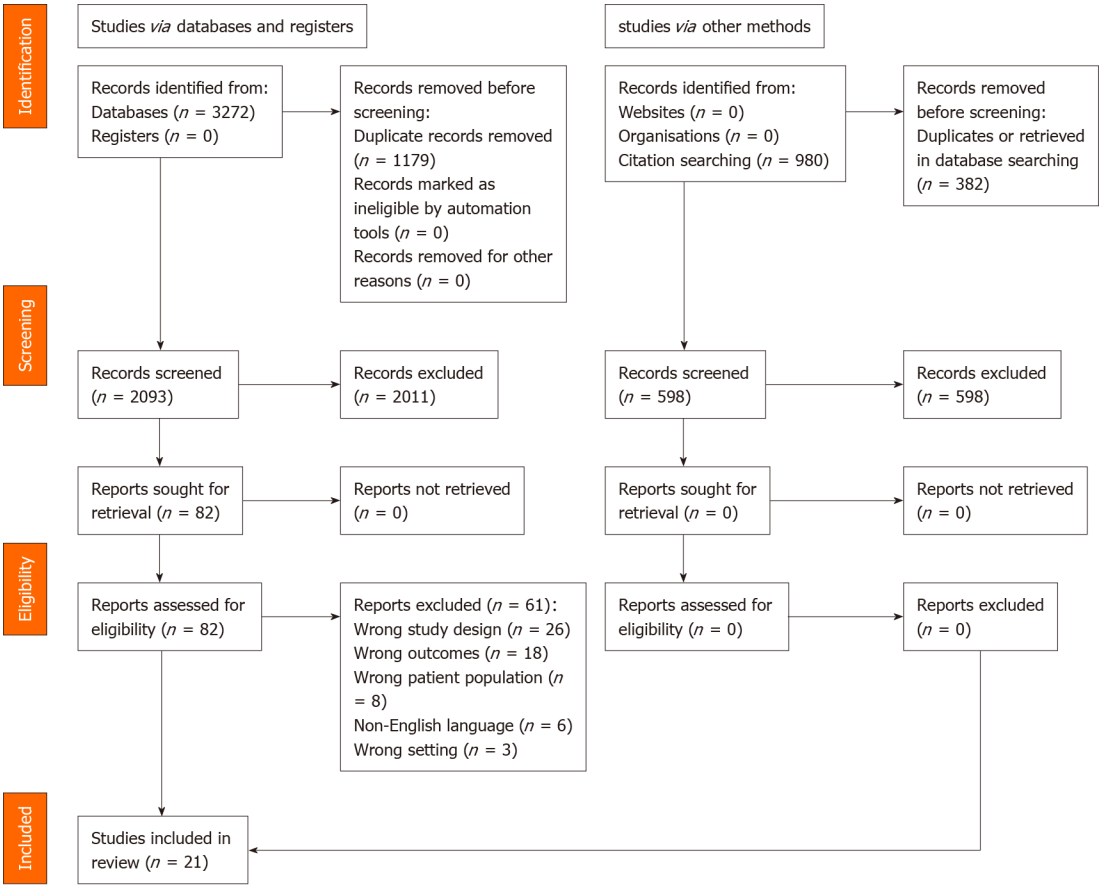


Figure 1 PRISMA flow diagram describing study selection.

Prevalence of osteoporosis among CP patients: The pooled prevalence of osteoporosis in CP patients was 20.9% (95%CI: 14.9%-27.6%; $P < 0.0001$; $\chi^2 = 290.33$, $df = 18$, $P < 0.0001$; $I^2 = 93.8\%$, $\tau^2 = 0.1$)[7,8,17-19,21-23,25-35] (Figure 2B). The pooled prevalence of osteoporosis in CP patients had high heterogeneity since $I^2 > 75\%$.

Prevalence of fragility fracture among CP patients: The pooled prevalence of fragility fracture was 5.9% (95%CI: 3.9%-8.4%, $P < 0.0001$; $\chi^2 = 186.0$, $df = 8$, $P < 0.0001$; $I^2 = 95.7\%$, $\tau^2 = 0.02$)[7,19-21,24,26,32,34,35] (Figure 2C). All of the studies prevalence estimates had high heterogeneity since $I^2 > 75\%$.

Factors impacting bone disease in CP patients: Various covariates underwent qualitative assessment for their association with CP related bone disease (Table 3).

Baseline patient characteristics: Female population was excluded in three studies[17,22,28] whereas 6376 (31.6%) female subjects were incorporated in the remaining 18 studies. In addition, three studies excluded post-menopausal females[19,25,33]. The race of CP patients was described in only five studies [7,20,24,33,34]. Its association with bone disease was investigated only by Hart and colleagues and they observed significantly higher burden among Caucasian population[34].

CP activity: The impact of CP severity on the bone outcomes was investigated in only one third of the eligible studies[17,19,27,28,31,32,34]. These studies observed no correlation with CP severity and alteration in bone mineral density. Moreover, the association of bone disease with duration of CP was also studied and lacked statistically significance[18,29,32,33,34,35]. The studies by Sikkens *et al*[25], and Kuhlman *et al*[30], observed a significant association of bone disease with exocrine insufficiency. Improved bone disease outcomes were observed in patients receiving PERT based on four large sized studies[8,26,30,34]. PERT usage and CP-related bone disease lacked statistical significance in five observational studies[17,22,23,28,32].

Inflammatory factors: Inflammation-based biomarkers such as C-reactive protein and interleukin (IL)-6 levels also yielded non-significant results in the investigation by two groups[27,32].

Endocrine factors: Among the eligible studies, hormones which regulate calcium metabolism have been widely studied. Serum 25-OH cholecalciferol had a positive correlation with bone density in three

Table 3 Characteristics of various covariates and their association with outcome measures

Ref.	PERT use, *n* (%)	Inflammatory markers: CRP/IL-6, mean ± SD	Vitamin D deficiency, *n* (%)	Serum PTH, mean ± SD	Alcohol exposure, *n* (%)	Smokers, *n* (%)	Diabetes, *n* (%)	Nutritional parameters	Relevant covariates findings
Morán *et al* [17], 1997	CP: 4 (28.57) Controls: -	CP: - Controls: -	CP: 7 (50) Controls: -	CP: - Controls: -	CP: 0 Controls: -	CP: - Controls: -	CP: - Controls: -	Mean serum albumin 3.8 g/dL, 4 (28.6) had BMI < 20. Non-significant associations between osteopathy and BMI	Non-significant associations between osteopathy and (1) CP severity (as per fecal fat or bicarbonate secretion assessments); (2) CP etiology; (3) Age; and (4) Vitamin D, PTH or calcium
Haaber *et al* [18], 2000	CP: 26 (44.82) Controls: -	CP: - Controls: -	CP: - Controls: -	CP: 40[1] (31) Controls: -	CP: - Controls: -	CP: - Controls: -	CP: - Controls: -		Non-significant associations between osteopathy and (1) Duration of CP; and (2) Vitamin D and PTH
Dujsikova *et al* [19], 2008	CP: - Controls: -	CP: - Controls: -	CP: 63 (86.3) Controls: -	CP: - Controls: -	CP: 8 (10.95) Controls: -	CP: - Controls: -	CP: - Controls: -		Non-significant associations between osteopathy and severity of disease
Tignor *et al* [20], 2010	CP: - Controls: -	CP: - Controls: -	CP: - Controls: -	CP: - Controls: -	CP: - Controls: -	CP: - Controls: -	CP: - Controls: -		No descriptions of regression analysis or covariate adjustment
Sudeep *et al* [22], 2011	CP: - Controls: -	CP: - Controls: -	CP: 16 (51.6) Controls: -	CP: - Controls: -	CP: - Controls: -	CP: - Controls: -	CP: - Controls: -	BMI correlated significantly with BMC (r = 0.426; P = 0.017). There was an inverse correlation between stool fat and BMC (r = -0.47; P = 0.03)	Non-significant associations between osteopathy and (1) EPI (as per 72-hour fecal fat); and (2) Vitamin D
Joshi *et al*[21], 2011	CP: 33 (45.83) Controls: -	CP: CRP < 0.32 (-) Controls: CRP < 0.32 (-)	CP: 62 (86.11) Controls: 85 (85) Significant association of Lumbar Z score with log vitamin D (beta: 0.274; P = 0.04)	CP: 43.38[1] (-) Controls: 84.87[1] (-)	CP: - Controls: -	CP: 7 (9.7) Controls: -	CP: 52 (72.2) Controls: -	Lumbar Z score was associated with BMI (beta: 0.276; P = 0.04), serum albumin was significantly lower in patients compared with controls [4.0 (0.6) *vs* 4.6 (0.7) g/dL, P < 0.001]	
Duggan *et al* [23], 2012	CP: - Controls: -	CP: - Controls: -	CP: - Controls: -	CP: - Controls: -	CP: 58 (93.5) Controls: 62 (93.9)	CP: 46 (74.19) Controls: 40 (60.6)	CP: - Controls: -	BMI < 20: low BMD: 15 (23.8) *vs* normal BMD 10 (1.1)	Higher T scores for the lowest age tertile (P = 0.003). Lower T-score for smokers (P = 0.002). Non-significant associations between T scores at any area and (1) CP severity; (2) EPI; and (3) Ssex
Sikkens *et al* [25], 2013	CP: 19 (47.5) Controls: -	CP: - Controls: -	CP: - Controls: -	CP: - Controls: -	CP: 1 (2.5) Controls: -	CP: 27 (67.5) Controls: -	CP: - Controls: -	A high BMI is predictive of a ''higher'' lowest T-score [Coeff: 0.58 (0.2); P = 0.003]	Significant association between osteopathy and untreated EPI (P = 0.013)
Prabhakaran	CP: -	CP: -	CP: 20 (19.41)	CP: 27.6	CP: 72 (69.9)	CP: -	CP: 39	-	Non-significant associations between osteopathy

et al[28], 2014				(39.8)			(37.86)		and (1) EPI (as per steatorrhea assessment); (2) CP severity; and (3) CP etiology
	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -		
			Non-significant associations between osteopathy and vitamin D, PTH and alkaline phosphatase						
Bang *et al*[26], 2014	CP: 3545 (29.61)	CP: -	CP: -	CP: -	CP: 3651 (30.49)	CP: -	CP: -		Increased risk of fracture among smokers (HR, 1.8; 95%CI, 1.7-1.8) and alcohol related CP (HR, 2.0 *vs* 1.5; $P < 0.0001$)
	Controls: -	Controls: -	Controls: -	Controls: -	Controls: 2753 (2.29)	Controls: -	Controls: -		
	Reduced fracture risk among PERT treated CP patients (HR, 0.8; 95%CI, 0.7-0.9)								
Duggan *et al*[27], 2015	CP: -	CP: CRP: 3.15 (-), IL-6: 5.61 (-)	CP: 20	CP: 47.1 (19.4)	CP: 27 (93.10)	CP: 23 (79.3)	CP: -	Lower T scores were associated with BMI ($P = 0.04$)	Lower T scores were associated with age ($P = 0.006$). Non-significant association with carboxy-terminal telopeptide of type I collagen; osteocalcin; Procollagen 1 amino-terminal propeptide
	Controls: -	Controls: CRP: 0.9 (-), IL-6: 3.58 (1.82)	Controls: 18	Controls: 46.3 (14)	Controls: 28 (96.55)	Controls: 10 (34.4)	Controls: -		
		Non-significant association with IL-6 and CRP	Lower T scores were associated with serum vitamin D ($P = 0.002$). No association with PTH						
Munigala *et al*[24], 2016	CP: -	CP: -	CP: -	CP: -	CP: 494 (15.16)	CP: 505 (15.5)	CP: -	A significant association of BMD in the columnar spine with vitamin D level (coefficient 0.13 g/cm^2; $P = 0.017$) and BMI (coefficient 0.14 g/cm^2 ; $P = 0.007$) were observed on univariate analysis	Increased fracture risk among males (adjusted OR, 1.73% (95%CI: 1.46%-2.05%); $P < 0.0001$), alcohol consumers (OR, 2.30), and smokers (OR, 1.97). Non-significant associations between osteopathy and age
	Controls: -	Controls: -	Controls: -	Controls: -	Controls: 37146 (8.24)	Controls: 77926 (17.29)	Controls: -		
Kumar *et al*[29], 2017	CP: -	CP: -	CP: 69 (67.64)	CP: -	CP: -	CP: -	CP: 54 (52.94)	A MUST score (malnutrition score) of 1 or higher was associated with an increased risk for osteopenia and osteoporosis on Fisher's exact test ($P = 0.0037$)	Non-significant association between osteopathy and duration of CP
	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -		
			Non-significant association between osteopathy and vitamin D						
Stigliano *et al*[32], 2018	CP: 116 (54.97)	CP: -	CP: 119 (56.39)	CP: -	CP: 127 (60.18)	CP: 145 (68.72)	CP: 77 (36.49)	Observed significant association of BMI with osteopathy (OR 0.89;	Osteopathy more prevalent with increasing age (OR 1.06; $P = 0.0002$), female sex (OR: 3.44; $P =$

	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	95%CI: 0.83-0.96; P = 0.003)	0.0005). Non-significant association between osteopathy and (1) CP severity; (2) EPI (as assessed by fecal elastase); (3) Smoking; (4) Duration of CP; and (5) Alcohol exposure
	Non-significant association between osteopathy and PERT usage	Non-significant association between osteopathy and IL-6/CRP	Non-significant association between osteopathy, vitamin D and PTH						
Kuhlmann *et al*[30], 2018	CP: 28 (41.79)	CP: -	CP: -	CP: -	CP: 42 (62.68)	CP: 42 (62.68)	CP: 22 (32.83)	The underweight BMI category, had significant higher odds of osteopathy (OR: 7.40; 95%CI: 1.56-34.99; P < 0.001)	Lower Z scores associated with (1) EPI (P = 0.01); (2) Smoking (P = 0.02). Non-significant association with alcohol exposure
	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -		
			Lower Z scores associated with vitamin D (P = < 0.001)						
Min *et al*[31], 2018	CP: -	CP: -	CP: -	CP: -	CP: -	CP: -	CP: -	Non-significant association with BMI	Non-significant association with (1) CP severity; (2) PERT usage
	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -		
Gupta *et al* [33], 2019	CP: -	CP: -	CP: -	CP: -	CP: 13 (34.21)	CP: 18 (47.36)	CP: 12 (31.57)		Low bone mass was associated with lower BMI. Non-significant association with CP duration
	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -		
Kanakis *et al* [7], 2020	CP: -	CP: -	CP: -	CP: -	CP: 130 (54.39)	CP: 132 (55.23)	CP: -	For patients, there was no association between total hip BMD and BMI (P = 0.753)	No descriptions of regression analysis or covariate adjustment
	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -		
Hart *et al*[34], 2021	CP: 161 (57.09)	CP: -	CP: -	CP: -	CP: -	CP: 191 (67.7)	CP: 111 (39.36)	Higher osteopathy risk associated with low BMI (P ≤ 0.001)	Increased risk of osteopathy with white race (P = 0.017), age (P ≤ 0.001), female sex (P ≤ 0.01) and past or present smoking (P ≤ 0.01). No associations with (1) CP severity (per atrophy assessment); and (2) Duration of CP
	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -		
	Reduced osteopathy among PERT users (P = 0.02)								
Vujasinovic *et al*[8], 2021	CP: 104 (88.13)	CP: -	CP: -	CP: -	CP: 53 (44.91)	CP: 76 (64.4)	CP: 28 (23.72)		
	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -		
	Reduced time to first fracture in PERT-treated patients								
Tang *et al*[35], 2021	CP: 51	CP: CRP: 0.75 (-), IL-6: 4.51 (-)	CP: 76 (73.07)	CP: 40.86 (-)	CP: 52 (50)	CP: 45 (43.26)	CP: 28 (26.92)	Independent predictors of osteopathy: BMI (OR, 0.72; 95%CI, 0.58-0.89; P = 0.003)	Non-significant association between osteopathy and (1) Age; and (2) Duration of CP
	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -		
		Non-significant	Non-significant						

	association between osteopathy and IL-6/CRP	association between osteopathy and PTH

[1]Number of subjects who consented to Dual-energy X-ray Absorptiometry scan if detailed in the studies.

CP: Chronic pancreatitis; EPI: Exocrine pancreatic insufficiency; PTH: Parathyroid; PERT: Pancreatic enzyme replacement; BMI: Body mass index; CRP: C-reactive protein; IL-6: Interleukin 6.

studies[21,27,30], whereas the remaining reported no significant associations[17,18,22,28,29,32]. Similarly, alkaline phosphatase and calcium had non-significant correlation with bone mineral density in two studies[17,28]. Serum PTH level did not correlate with bone disease in three studies[17,18,28], in contrast to the significant association found by Stigliano *et al*[32], Duggan *et al*[27], and Tang *et al*[35]. Novel bone turnover-based biomarkers *i.e.*, carboxy-terminal telopeptide of type I collagen, osteocalcin, procollagen 1 amino-terminal propeptide also had non-significant results in limited observational studies[27,35]. Thyroid-stimulating hormones and Insulin-like growth factors 1 were considered by Munigala *et al*[24], but no correlation was found with bone mineral density (BMD). Although hypogonadism was higher in subjects with low BMD in the study by Gupta e*t al*[33], no statistically significant difference was found. Exogenous hormone uses or replacement therapy was not investigated in any of the included studies.

Nutritional factors: The evidence linking BMI with bone disease outcomes has been conflicting. Whereas six studies described higher BMI as a protective factor[19,21-23,30,32,35], others reported an increased risk or non-significant findings[17,31,33,34]. Serum albumin was also explored in two studies with no mention of outcome association[17,21]. Min and colleagues studied the Malnutrition Universal Screening Tool, a validated nutritional assessment tool and observed that a score of 1 or more had significant association with osteopenia and osteoporosis ($P = 0.004$)[31]. Three studies described calcium supplement intake but did not study its relationship with bone disease[21,22,30].

Lifestyle factors: The definitions for exposure to alcohol and/or smoking were heterogeneous with limited evidence on their impact on bone outcomes[7,8,17,19,21,23-28,30,32-35]. Outdoor activity and sunlight exposure was only investigated by Joshi *et al*[21], and although correlated positively with vitamin D levels, their impact on osteoporosis was not studied.

Meta-regression

Meta-regression revealed significant association of PERT use with prevalence of osteoporosis [coefficient: 1.7 (95%CI: 0.6-2.8); $P < 0.0001$] but not with osteopenia or fragility fractures. No associations with mean age, sex distribution, BMI, alcohol or smoking exposure, vitamin D deficiency, PTH levels, and diabetes with prevalence of osteopenia, osteoporosis or fragility fractures (Table 4).

Sensitivity analysis

Sensitivity analysis through forest plot calculation was performed using the "remove one study" method as described above. Confidence intervals and heterogeneity was measured through I^2 and no significant alterations were observed after removal of one particular study (Supplementary Table 4).

Table 4 Metaregression of outcome measures with various covariates

Covariates	Osteopenia		Osteoporosis		Fragility fracture	
	95%CI	*P* value	95%CI	*P* value	95%CI	*P* value
Sex distribution	0.2 (-1.2-1.5)	*P* = 0.8	0.5 (-1.9-2.9)	*P* = 0.7	0.3 (-3.2-3.7)	*P* = 0.9
Age	0.0 (0-1)	*P* = 0.6	0.0 (-0.1-0.0)	*P* = 0.3	0.0 (-0.5-0.4)	*P* = 0.7
DM	0.3 (-5.5-6.0)	*P* = 0.9	0.0 (-3.9-3.8)	*P* = 0.9	-13.8 (-107.4-79.8)	*P* = 0.3
Alcohol use	0.0 (-1.5-1.3)	*P* = 0.1	0.8 (-8.5-10.0)	*P* = 0.7	-0.5 (-5.4-4.4)	*P* = 0.3
Vitamin D	0.0 (-0.02-0.0012)	*P* = 0.4	-0.002 (-0.004-0.001)	*P* = 0.2	0.0 (-0.003-0.005)	*P* = 0.5
PTH levels	0.002 (-0.02-0.02)	*P* = 0.5	0.0 (-0.02-0.03)	*P* = 0.4	-	-
PERT	1.7 (0.6 -2.8)	*P* = 0.2	1.7 (0.6-2.8)	*P* < 0.0001	1.0 (-4.3-6.2)	*P* = 0.5
Smoking	0.0 (-3.6-3.6)	*P* = 0.9	-0.5 (-2.9-1.9)	*P* = 0.6	2.0 (-3.4-7.9)	*P* = 0.3
Mean BMI	NA	*P* = 0.9	0.0 (-0.12-0.21)	*P* = 0.6	0.5 (-17.2-18.2)	*P* = 0.8

DM: diabetes mellitus; PTH: Parathyroid; PERT: Pancreatic enzyme replacement; NA: *P* value calculated through Monte Carlo permutation, coefficient not available; -: Insufficient values.

Study quality and publication bias assessment

All the observational studies were assessed for quality in selection, comparability, and outcome/exposure domain and their scores ranged from 5-8 and were in good agreement (93% agreement, Cohen's κ = 0.87) (Supplementary Table 5). The comparability domain was variable based upon the presence or absence of controls. Visual inspection of funnel plot and Egger's regression for studies describing osteopenia ($P = 0.003$), and osteoporosis ($P < 0.001$) demonstrated significant publication bias (Figure 3).

DISCUSSION

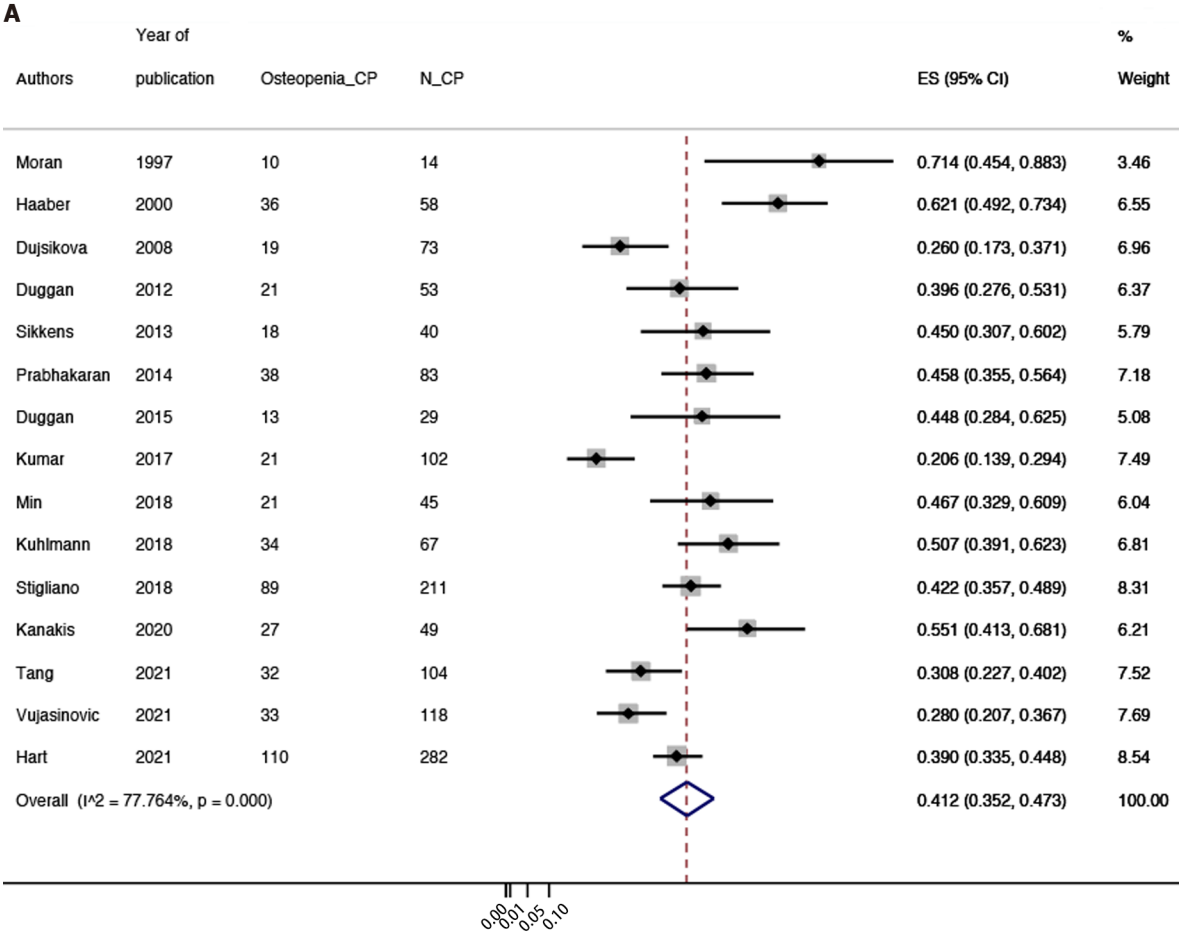
Through this systematic review we present the burden of osteopenia, osteoporosis, and fragility fractures in CP patients. The majority of the studies used bone mineral density to quantitate bone disease, which is a validated tool for use in the general population[36]. Our meta-analyses revealed osteopenia and osteoporosis to be prevalent in 41.2% and 20.9% of the CP population, respectively. We also observed a fracture risk of 5.9% among CP patients, which includes low trauma fractures predominantly in the hip, vertebrae and distal radius. These may cause pain, falls, and hospitalizations which may significantly impair the lives of CP patients along with pre-existing morbidities like malabsorption and chronic abdominal pain[37].

Although dedicated studies among CP patients are lacking, reduced bone mass and resultant fractures cause significant pain, reduced functionality, and quality of life in both men and women[38]. Chronic inflammatory conditions affecting the gastrointestinal tract, such as inflammatory bowel disease, celiac disease and chronic liver disease have been known to disrupt the balance between bone resorption and formation and cause bone disease. Histomorphometric analysis among alcohol related CP patients has revealed low cortical and trabecular bone thickness and endocortical apposition and growth rate as compared to controls[39]. Similarly, increased bone turnover and mineralization defects due to malnutrition have been observed in CP[27,40]. Although recent CP guidelines in the United States and Europe have suggested surveillance of bone-related disorders, studies report poor adherence to these guidelines, suggesting CP-related bone disease is an underappreciated clinical entity[7,8].

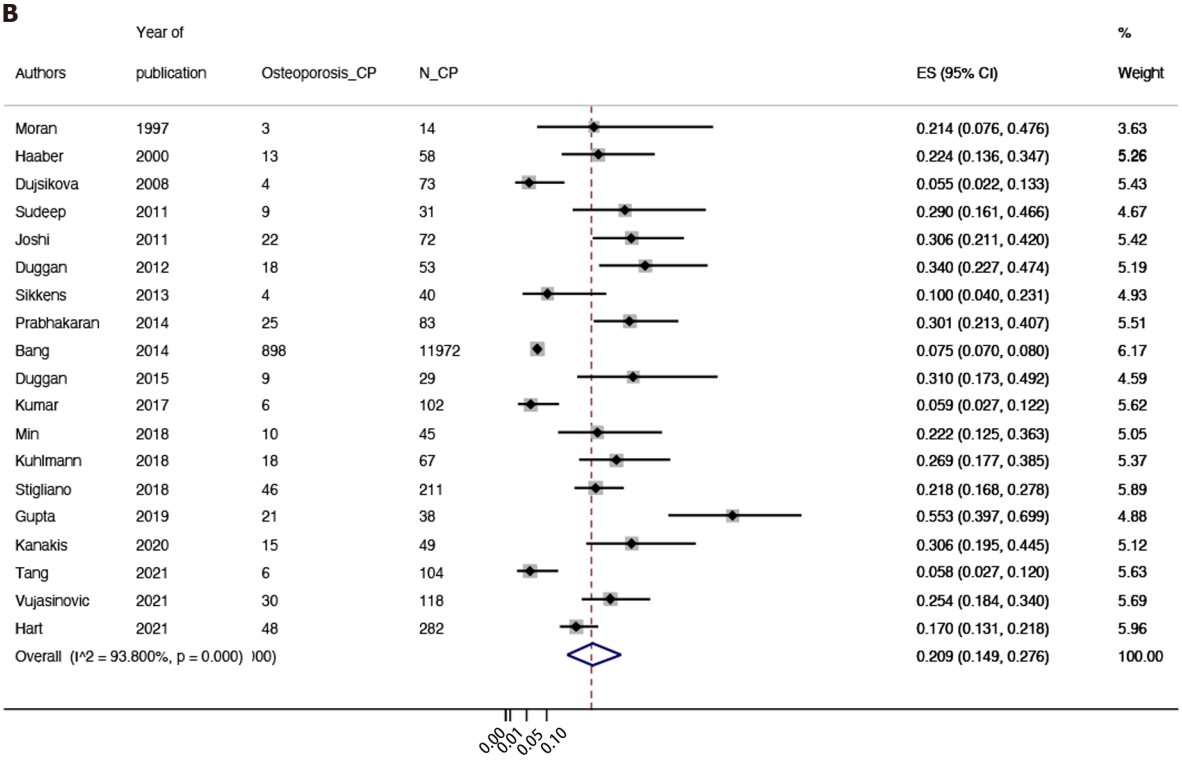
CP-related bone disease has been quantified previously by Duggan *et al*[9] in 2014 who observed similar prevalence of osteopenia and osteoporosis *i.e.*, 39.8% and 23.4%, respectively. Since this publication, over the past decade, several additional observational studies have investigated bone mineral density and metabolism in CP patients[7,8,24,25,27-31,33,34], and therefore, provide a unique opportunity to further investigate the evidence in this clinical domain. Hence, we appraised all the available literature, including the most recent publications, in an effort to contribute evidence for future by also uniquely quantifying fragility fractures which are significant complications of CP-related bone disease.

The current systematic review is powered by high-quality studies with large sample size and clearly defined study population and outcome measures. In addition, strengths of the study include a clearly articulated a priori analysis plan, a thorough search strategy and a conservative analysis. However, our study is limited by variability in the CP definitions from centers worldwide and spanning over 2

A


Authors
Year of publication
Osteopenia_CP
N_CP
ES (95% CI)
%
Weight
Moran 1997 10 14 0.714 (0.454, 0.883) 3.46
Haaber 2000 36 58 0.621 (0.492, 0.734) 6.55
Dujsikova 2008 19 73 0.260 (0.173, 0.371) 6.96
Duggan 2012 21 53 0.396 (0.276, 0.531) 6.37
Sikkens 2013 18 40 0.450 (0.307, 0.602) 5.79
Prabhakaran 2014 38 83 0.458 (0.355, 0.564) 7.18
Duggan 2015 13 29 0.448 (0.284, 0.625) 5.08
Kumar 2017 21 102 0.206 (0.139, 0.294) 7.49
Min 2018 21 45 0.467 (0.329, 0.609) 6.04
Kuhlmann 2018 34 67 0.507 (0.391, 0.623) 6.81
Stigliano 2018 89 211 0.422 (0.357, 0.489) 8.31
Kanakis 2020 27 49 0.551 (0.413, 0.681) 6.21
Tang 2021 32 104 0.308 (0.227, 0.402) 7.52
Vujasinovic 2021 33 118 0.280 (0.207, 0.367) 7.69
Hart 2021 110 282 0.390 (0.335, 0.448) 8.54
Overall (I^2 = 77.764%, p = 0.000) 0.412 (0.352, 0.473) 100.00
0.00 0.01 0.05 0.10

B


Authors
Year of publication
Osteoporosis_CP
N_CP
ES (95% CI)
%
Weight
Moran 1997 3 14 0.214 (0.076, 0.476) 3.63
Haaber 2000 13 58 0.224 (0.136, 0.347) 5.26
Dujsikova 2008 4 73 0.055 (0.022, 0.133) 5.43
Sudeep 2011 9 31 0.290 (0.161, 0.466) 4.67
Joshi 2011 22 72 0.306 (0.211, 0.420) 5.42
Duggan 2012 18 53 0.340 (0.227, 0.474) 5.19
Sikkens 2013 4 40 0.100 (0.040, 0.231) 4.93
Prabhakaran 2014 25 83 0.301 (0.213, 0.407) 5.51
Bang 2014 898 11972 0.075 (0.070, 0.080) 6.17
Duggan 2015 9 29 0.310 (0.173, 0.492) 4.59
Kumar 2017 6 102 0.059 (0.027, 0.122) 5.62
Min 2018 10 45 0.222 (0.125, 0.363) 5.05
Kuhlmann 2018 18 67 0.269 (0.177, 0.385) 5.37
Stigliano 2018 46 211 0.218 (0.168, 0.278) 5.89
Gupta 2019 21 38 0.553 (0.397, 0.699) 4.88
Kanakis 2020 15 49 0.306 (0.195, 0.445) 5.12
Tang 2021 6 104 0.058 (0.027, 0.120) 5.63
Vujasinovic 2021 30 118 0.254 (0.184, 0.340) 5.69
Hart 2021 48 282 0.170 (0.131, 0.218) 5.96
Overall (I^2 = 93.800%, p = 0.000) 0.209 (0.149, 0.276) 100.00
0.00 0.01 0.05 0.10

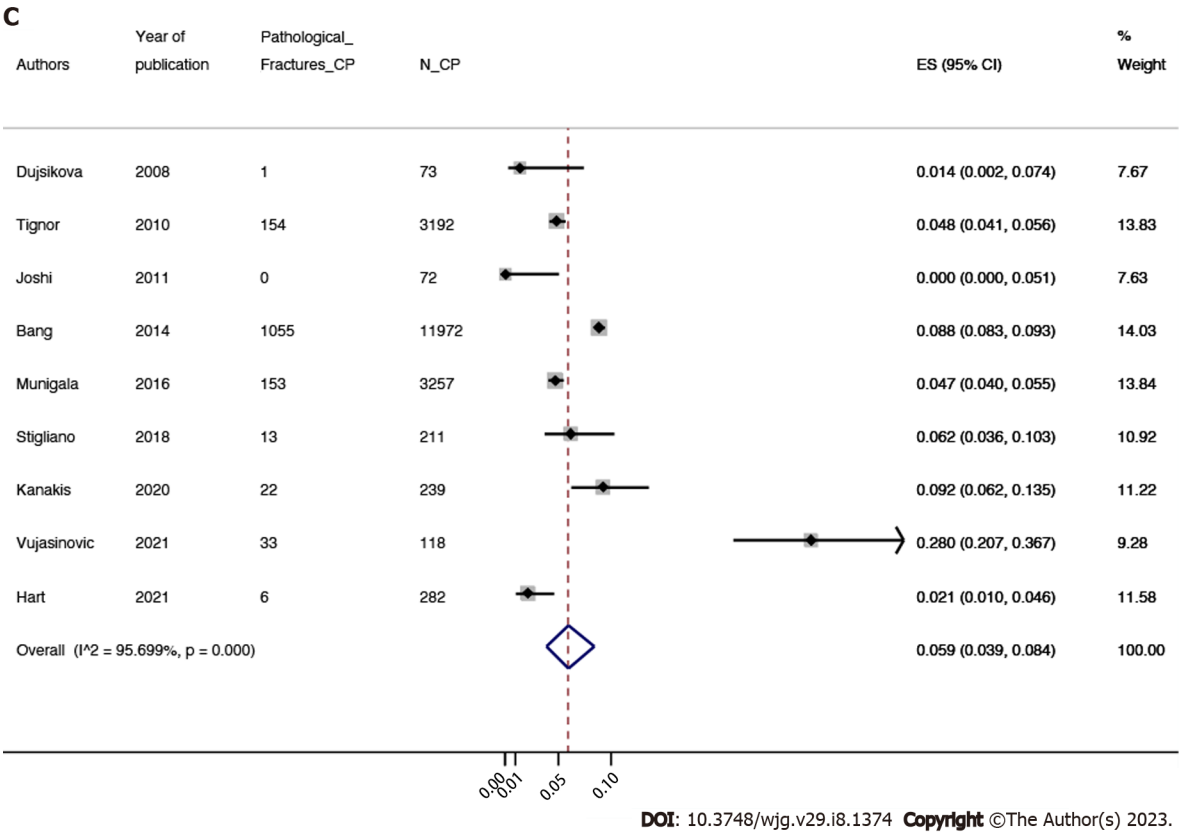
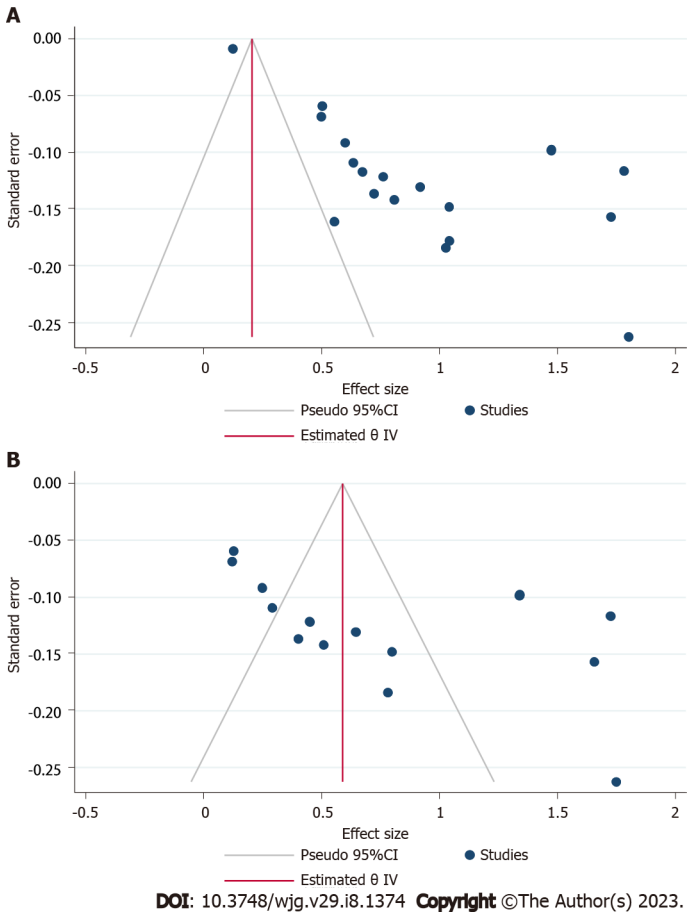


Figure 2 Forest plot demonstrating pooled prevalence of bone disease in chronic pancreatitis. A: Forest plot demonstrating pooled prevalence of osteopenia in chronic pancreatitis (CP); B: Forest plot demonstrating pooled prevalence of osteoporosis in CP; C: Forest plot demonstrating pooled prevalence of fragility fractures in CP. ES: Effect size; N: Total chronic pancreatitis population.

decades. Besides inconsistencies in definitions, high heterogeneity in the effect sizes is evident from I^2 mandated performance of metaregression and sensitivity analysis. The heterogeneity was not attributable to any specific study as demonstrated by sensitivity analysis. We observed significant association of osteoporosis with patients with PERT use on metaregression analysis. Although PERT use signifies severe disease, this association doesn't not establish causality and this in conjunction with limited information on the dosage of PERT or nutritional outcomes prevent substantial inferences to be drawn from this association[41]. We acknowledge significant publication bias, and a priori exclusion of studies in foreign language and conference abstracts[42]. These limitations prevent making categorical recommendations to patients.

Various mechanisms have been hypothesized to cause CP-mediated bone disease. Risk factors for CP such as cigarette smoking and alcohol exposure have been proven to alter the PTH-vitamin D axis and gonadal hormones and cause oxidative stress[39,43-46]. This clinical entity is also hypothesized to be driven by RANK ligand-induced osteoclastogenesis typically stimulated by inflammation-mediated nuclear factor-kappa B ligand[47]. Prior studies have also evaluated the relation of CP with inflammatory markers, such as IL-6, IL-1, and tumor necrosis factor-alpha[48]. Protein malnutrition lowers bone mass whereas deficiency of fat-soluble vitamins contributes to defects in mineralization and thus causes osteoporosis and resultant stress fracture[49,50]. CP is also characterized by low skeletal muscle, weight loss, and low mobility, all of which negatively impacts bone mass[51-53].

CP-related bone disease warrants further investigation to answer a few clinical queries. While CP patients are at risk, the impact of disease severity and duration on bone outcomes are unknown. Additionally, poor correlation between clinical symptomatology, severity and imaging findings in CP patients presents challenges for research[54]. We also systematically assessed various confounders including baseline clinical features and impact of bone turnover and inflammatory markers, albeit our results were unremarkable. The evidence on calcium supplements, hormone levels and outdoor activity among included studies was lacking. The studies that investigated mechanisms of systemic inflammation, bone turn over and malabsorption were underpowered, whereas those pertaining to vitamin D had conflicted evidence[17,18,21,22,27-30,32]. Among this at-risk group, efficacy of preventative therapy for osteoporotic fracture, drug interactions, and adverse effect also remain elusive.



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Figure 3 Funnel plots showing the publication bias assessment in studies describing patients with osteopenia and osteoporosis. A: Osteopenia ($Z = 2.9$, $P = 0.003$); B: Osteoporosis ($Z = 4.7$, $P < 0.001$).

CONCLUSION

In summary, this meta-analysis confirms significant bone disease in patients with CP. We observed significant association of PERT with CP related osteoporosis. Our study calls for improved methodology dedicated at delineation of confounders and studies targeting identification of at-risk CP patients, deeper understanding of mechanisms of CP related bone disease and their implications of treatment response. Fragility fractures are an important consequence of bone disease, which we have found to be increased in patients with CP. Screening strategies in this at-risk population with CP are needed as well as evaluating quality of life due to consequences of bone disease.

ARTICLE HIGHLIGHTS

Research background

Chronic pancreatitis (CP) is a multifactorial fibro-inflammatory syndrome is characterized by nutritional deficiencies, systemic inflammation, and etiological factors like alcohol or smoking may disrupt the balance between bone formation and resorption. These maladaptive alterations may cause osteopenia and osteoporosis, resulting in fragility fractures which result from low energy trauma and have significant implications on quality of life.

Research motivation

Multiple large-sized studies on CP patients including those from multicenter cohorts have investigated the burden of bone disease in CP as well as their association with nutritional, anthropometric, and inflammatory parameters. Thus, with quantitative data on covariates and fragility fractures available, a synthesis of evidence is pertinent.

Research objectives

This systematic review and meta-analysis sought to quantify the prevalence of osteopenia, osteoporosis,

and fragility fractures in CP patients and delineate clinical parameters which impact their occurrence.

Research methods

The study included systematic review and then metanalysis of studies describing bone disease in CP patients. A preregistered systematic search enabled identification of original studies from Cochrane Library, Embase, Google Scholar, Ovid Medline, PubMed, Scopus, and Web of Science, from inception until October 2022. The metanalysis was performed using random effect model and the outcomes of interest included prevalence of osteopenia, osteoporosis, and fragility fractures. To assess the association of these outcomes with covariates metaregression using random effect model was performed.

Research results

Twenty-one studies were included for systematic review and 18 studies were included for meta-analysis. The pooled prevalence of osteopenia and osteoporosis in CP patients was 41.2% (95%CI: 35.2%-47.3%) and 20.9% (95%CI: 14.9%-27.6%), respectively. The pooled prevalence of fragility fractures described among CP was 5.9% (95%CI: 3.9%-8.4%). Meta-regression showed no associations of bone outcomes in CP patients with mean age, sex distribution, body mass index, alcohol or smoking exposure, diabetes, serum parathyroid levels, and vitamin D deficiency with prevalence of osteopenia, osteoporosis or fragility fractures. A significant association of pancreatic enzyme replacement therapy use with prevalence of osteoporosis [coefficient: 1.7 (95%CI: 0.6-2.8); $P < 0.0001$]. Due to sparse data on systemic inflammation, CP severity, and bone mineralization parameters a formal meta-analysis was not feasible.

Research conclusions

This meta-analysis confirms significant bone disease: Osteopenia, osteoporosis, and fragility fractures in CP patients. Although pancreatic enzyme use had significant association with osteoporosis, the link between osteopathy and various patient or study-specific factors remains unclear. Further investigation is needed for delineation of at-risk population, to understand the mechanisms of CP-related bone disease, and assess the therapeutic response to treatment modalities.

Research perspectives

Our study calls for dedicated studies targeting delineation of confounders and identification of at-risk features in CP patients. There is also a knowledge gap in therapeutic response among CP patients with bone disease.

FOOTNOTES

Author contributions: Sheth SG and Chhoda A contributed to the study conception; Chhoda A, Addo NAA, and Hernandez-Woodbine MJ contributed to the study selection; Chhoda A and Hernandez-Woodbine MJ contributed to the data acquisition, analysis, and manuscript drafting; Grimshaw A contributed to the study accrual; Gunderson C contributed to the statistical supervision; Chhoda A and Nasir SA contributed to the study quality assessment; Ahmed A, Freedman SD, and Sheth SG contributed to the manuscript edition.

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PRISMA 2009 Checklist statement: The authors have read PRISMA checklist and revised according to PRISMA checklist.

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Country/Territory of origin: United States

ORCID number: Ankit Chhoda 0000-0002-6232-0590; Maria Jose Hernandez-Woodbine 0000-0002-1481-617X; Alyssa Grimshaw 0000-0003-3985-0056; Steven D. Freedman 0000-0003-1255-9701; Sunil G. Sheth 0000-0003-0602-8509.

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